FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
UNIVERSITY OF HELSINKI

STUDIES IN ANTIBIOSIS BETWEEN THE GENERA ESCHERICHIA AND MICROCOCCUS

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ERRATA

On page 11, line 21, anthrax bacilli should be anthrax

On page 26, lines 36 and 38, epidermis should be epidermidis

On page 56, line 30, Section I should be Section 1

On page 68, line 31, epidermis should be epidermidis

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PREFACE

The present work was carried out in 1953—1955 at the Department of Serology and Bacteriology, University of Helsinki.

I wish to extend my warmest thanks to the head of the Institute, Prof. K. O. Renkonen, M. D., who supervised my investigations into the antibiotic and antagonistic phenomena among bacteria, for his inspiring interest and for the encouraging, and also critical, remarks with which he followed my work throughout. During the eight years I have worked in the Institute, I have learned to know him as a skilful and admirable teacher, who always has time available for his pupils, from the most junior to the most senior, and for their problems.

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M. E. P.

INTRODUCTION

In the upper part of the small intestine the predominant flora is the enterococci, to which are added, in the more distal parts, many Gram-negative rods, micrococci, various organisms decomposing gelatine, and yeast fungi. In the large intestine the flora becomes still more variable, although the vast majority consists of both aerobic and anaerobic Gram-negative rods. In addition, there are plenty of Gram-positive, spore-forming anaerobes, lactobacilli and enterococci, and smaller quantities of various spore-forming aerobic rods, various micrococci, yeasts, spirochetes, etc.

In normal conditions the proportions of the different species remain roughly the same in a person staying and taking his meals in the same milieu. When the person moves into another milieu, variations may occur in the types of the different species, but the overall picture remains practically unchanged. In pathological conditions, by contrast, the equilibrium of the flora is disturbed for various reasons, and a species which has previously held a subordinate position, or even a completely new species introduced from elsewhere may become the dominant flora.

Previously, when no selective culture media were available, it was found, and still is found today when ordinary culture media are used in aerobic conditions, that Gram-negative rods and enterococci constituted the bulk of the colonies in faecal cultures. For instance, Schiller 1913 (204) failed to find Micrococcus pyogenes v. aureus at all in the faeces of normal cases, and found albus strains in only 2/12ths of the cases. Using selective culture medium Chapman (33) found aureus in 12 out of 124 permanently handicapped persons (9.2 per cent of the cases). On the other hand, Linz (155) found pathogenic micrococci in the faeces of 38 per cent of patients suffering from enteritis. Infants with diarrhoea, according to Rantasalo (169), often show plenty of pathogenic micrococci in the faeces.

If the equilibrium of the normal flora is disturbed by other factors, such as antibiotics, it is possible that more micrococci will be found than normally. For instance, cultures taken from autopsies by Oeding and coll. (182) showed M. pyog. v. aureus in 26 per cent of the patients treated with antibiotics but in only 17 per cent of those who had received no such treatment. The broad-spectrum antibiotics in particular produce considerable changes in the intestinal flora (46, 48), the strains sensitive to them disappearing while those resistant to them, which perhaps were not previously observed in the faeces because of their small numbers, become the predominant flora (207). Thus, M. pyogenes v. aureus also may be found in the faeces after antibiotic treatment even as a pure culture (60). Since antibiotics have become widely used as a preoperative therapy in intestinal surgery, the incidence of enterocolitis caused by the aureus varieties, sometimes even with fatal outcome, has been found to increase (19, 47, 60, 231).

It is obvious that antagonistic factors play a considerable part in the equilibrium of the microbial flora of the intestine. As the rods of the genus Escherichia have been found to possess antagonistic qualities, and they constitute the vast majority of the aerobic intestinal flora, further investigations into the antagonism between the genera Escherichia and Micrococcus seemed to be called for.

I REVIEW OF LITERATURE

EARLY HISTORY

Antagonism was discovered in practice long before the era of Pasteur. It is claimed (142) that the ancient Greek Thucydides had already noticed that other infections were cured during an epidemic. The phenomenon was therapeutically utilized as early as 1852 when Mosse, an English physician (68), treated boils with yeasts. The credit for the first real observation of the antagonism between two micro-organisms belongs, however, to Pasteur and Joubert (187) who found that certain "common bacteria" inhibited the reproduction of the anthrax bacillus in the urine. Garre (89) showed, ten years later, that antagonism may be bilateral or only unilateral. He found in vitro experiments that gelatin culture, in which B. fluorescens had been grown previously, failed to grow "staphylococcus", S. typhi or pneumococcus, whereas B. fluorescens was incapable of growing only on gelatin where S. typhi had grown before. In the same year, Emmerich (59) found that erysipelas infection inhibited the development of anthrax in vivo although the streptococcus causing it showed no antagonistic effect on the causative agent of anthrax bacilli in vitro. One year later de Freudenreich (84) studied the broth culture filtrates of several bacteria and found that many filtrates inhibited the development of a homologous and others also that of heterologous strains.

Lewek (154) found that E. coli weakened the growth of a separate, but closely situated "staphylococcal colony" which remained abnormally small. de Giaxa (93) observed in 1889 that sea water taken close to the outflow of sewers inhibited the reproduction of the causative agents of cholera, anthrax, typhoid fever and of the "staphylococcus" and that the first two even disappeared after a short time. When the water was sterilized by

heating the reproduction was normal. In the same year Charrin and Guignard (34) found that B. pyocyaneus destroyed in vitro the Bacillus anthracis, so that involution forms of B. anthracis began to develop after only six hours of mixed growth, and its virulence dropped. They attributed this not only to the metabolic products of B. pyocyaneus, but also to the loss of nutrients. In the same year Boughard (28) found that simultaneous inoculation of B. pyocyaneous protected a number of the guinea-pigs and rabbits employed as experimental animals from anthrax. Von Dunger (56) found that "B. friedländer" when alive, but to some extent also when dead, inhibited the development of anthrax in the rabbit, not by its direct effect on the causative agent of anthrax but due to its propagating effect on the white cells and hence on phagocytosis. Gosio (101), in 1896, isolated from a Penicillium species a crystalline substance which inhibited the development of Bacillus anthracis in vitro. The earliest detailed investigations into antagonistic substances were carried out by Lode (158) on Diplococcus, a colony of which was present by coincidence on a plate of M. tetragenus culture and extensively inhibited the growth of the M. tetragenus, and also on metabolites isolated from the diplococci.

MANIFESTATION AND FORMS OF ANTAGONISM

In compliance with Garre's (89) observation according to which the antagonism may be either unilateral or bilateral, all possible alternatives can be shown to exist in the interaction of microbes. Two of them may grow independently, increase or inhibit the growth of each other (224). Increase and inhibition may be unilateral or bilateral, and in certain situations, e.g. on solid culture media, both increase and inhibition may occur simultaneously (176, 224). In liquid culture media the antagonism may be temporary in two ways; either the originally inhibited strain will later grow freely and attain a normal concentration (86, 99) or the different strains will dominate alternately at different stages of growth (215). The occurrence of antagonism may also be facultative, depending on the environmental conditions, either the culture medium (145, 202, 220) or temperature (135); may be present only in vitro (213) or only in

vivo (56, 59) [in the later case, however, only growth inhibition due to non-specific stimulation (30) may be involved]; or it may be independent of the conditions, and persist all the time (145). Antagonism may be directed to homologous strains too. This so-called is-antagonism has been shown to occur in all bacteria (32, 35, 40, 58), and it has been considered responsible e.g. for the restriction of bacterial reproduction in liquid culture media, for instance, when so-called autotoxins are formed. Of greater importance, however, is the antagonism for other species, heteroantagonism, which is by no means limited only to the inhibition of the growth or destruction of other species, but is also connected with factors inactivating toxins (194) and with the destruction of growth-inhibiting factors produced by other species (225).

Garre (89) found in 1887 that putrefactive bacteria excreted metabolites which inhibited the growth of the cholera vibrio. A couple of years later Charrin and coll. (34) observed that the metabolites of pseudomonas inhibited the reproduction of the anthrax bacillus, although he assumed that loss of nutrients also contributed to it. Blagovetschensky (22) claimed in 1897 that the metabolites of Pseudomonas which inhibited the growth of other bacteria were volatile substances. Frost (85) showed in 1903 in his comprehensive and exceedingly accurate study of the antagonism of B. mesentericus for S. typhi that the antagonism cannot be caused by nutrient exhaustion, enzymes, pH changes or specific toxins but is due to the thermostabile metabolites produced by B. mesentericus which penetrate the colloid membrane. Ejkman (58) suggested in 1904 that all microbes excreted thermolabile, non-filtrable metabolites which have a greater effect on homologous than on heterologous strains. Many later investigators (e.g. 174, 208) also hold the view that various metabolites are involved. They may be undeterminable waste products (169), with a non-specific effect (64, 138) or with an effect only on homologous strains (193), or with both effects combined (2). Later research workers have made efforts to determine the type of these metabolites. Many investigators have studied substances similar to lipoids soluble in alcohol and ether. Pane (184), in 1910 found them in the alcohol extracts of e.g. Pseudomonas, "Staphylococcus" and pneumococcus

strains. Bogendörfer (23) found them in all microbes, their effect covering both homologous and heterologous strains. According to Gundel (113) and Gundel and coll. (117), however, they affect mainly the heterologous strains. The Pseudomonas lipoids, in particular, have been studied by very many investigators (15, 128). Similar substances have also been traced in yeasts (218). By further distillation of alcohol extracts, Komis (142) claimed to have obtained particularly effective bactericides, so-called apostaxins, from all bacterial species. — Water extracts, too, have been claimed to contain inhibiting sunstances (137). — Many strains form hydrogen peroxide (5, 125, 167), which on glucose-containing culture medium inhibits the growth of many bacteria, even those forming catalase, unless the medium itself contains catalase, e.g. blood. In 1946 Bonetti and coll, (24) showed that the antagonism of many micrococcal strains was due to peroxide formation, a finding later verified by many others (12, 110, 227). This group probably includes many strains believed to excrete specific inhibitors (13, 52, 162, 212). — Huga and coll. (129) showed in 1954 that the antagonistic effect of Micrococcus ureae, which decomposes urea, is due to the NH_3 it forms.

The most important group among the metabolites consists of the antibiotics, which are considered to possess a destructive effect primarily on the other species; in nature they are of importance in the first place in the struggle for existence between the species. Antibiotics were mentioned by Vuillemin (222) already in 1889, and subsequently Waksman (223) defined the term: "An antibiotic or an antibiotic substance is a substance, produced by micro-organisms, which has the capacity of inhibiting the growth and even of destroying other micro-organisms". The antibiotics are constitutional, usually crystallizable metabolites of no very complex structure, formed by micro-organisms independently of the environmental factors, i.e. the nature of the culture medium [an exception may occur, when e.g. certain aminoacids are absent (220)], or of the presence of a sensitive micro-organism.

As early as 1896, Gosio (101) isolated from penicillium mould a crystalline substance which had a growth-inhibiting effect on the anthrax bacillus. Due to the small amount of the substance

available, his studies were limited to this finding. In 1903 Lode (158) described in fairly great detail a diplococcus, which had arrived by chance on a plate culture of M. tetragenus and around the colony of which M. tetragenus failed to grow. He also studied the qualities, formation and antibiotic spectrum of the inhibitor. His experiments with animals failed, so that his investigations were limited to the one published report. In 1920, Pringsheim (191) described the antagonism of E. mesentericus. Fleming's (66) well-known penicillin studies were published in 1928. Among the pioneers, Dubos (49) and Vallee (219) may also be mentioned. At a later date it was observed that producers of specific antibiotics are to be found among practically all groups of micro-organisms (9).

A special group among the antibiotics consists of bacteriocines. In 1925 Gratia (104) found from an E. coli strain a lytic factor affecting another E.coli strain; it was later called the colicine (107). In his extensive series of investigations, Fredericq (75) showed that colicines consist of a group formed only by enterobacteria and acting only on enterobacteria. They have been found to be formed in vivo also (121). Similar groups have later been discovered among "staphylococci" (81, 122). The bacteriocines are very close to phages both in occurrence and mechanism (79), but differ from them at least on the following points (132): the bacteriocines resemble proteins, the phages nucleoproteins; the bacteriocines do not multiply in a sensitive strain as do the phages; the bacteriocines make their appearance just before, the phages after the latent stage of growth. Because of this close connection, the fourth edition of "Topley-Wilson", 1955 (216), assumes that the bacteriocines are not antibiotics but particles, similar to phages, representing forms of life simpler even than the viruses.

In his doctorial dissertation in 1903, Krenker (146) claimed that strains of Serratia and Proteus, in 4—6 week cultures, formed enzymes with an antagonistic effect on other strains. The enzymatic origin of antagonism was also assumed by Nicolle (179), and Neufeld (177); Ramon and coll. included the enzymes in the so-called antagonistic complex, a term by which they refer to a combination of several different factors leading to antagonism (195). Anzai and coll. (3) have shown

that in the antagonism of spore-forming bacteria, for instance, the antibiotic substance is bactericidal, and all that remains to the enzymes, the protamylases, is to digest the dead sensitive bacteria. Quite recently, Mathew and coll. (165) showed that the antagonistic effect of myxobacteria was due not only to the antibiotics proper, but also to the proteases produced by them.

Davide (44) showed that some pseudomonas and proteus strains, when growing on tubercle bacillus, which was their only source of nutrients, produced substances which killed the tubercle bacillus also in vivo; if an adapted pseudomonas or proteus strain was grown in ordinary broth, filtrable substances fatal to tubercle bacillus still developed in it (45). When grown in mixed populations these strains, whose filtrates primarily have no antagonistic effect, develop enzymes which have an antagonistic effect in the filtrate too (so-called adaptation antagonism) (96). The same adaptation-enzymatic antagonism is found in the antagonism of pseudomonas to the anthrax bacillus (133) and in that of the Penicillium and Aspergillus species to the Salmonellae (139). Adaptation antagonism is usually transient, since on separation from the sensitive strain the strains lose their ability to develop enzymes against it (97), but it can be permanent, when a mutation is in question (14).

Another special class is constituted by the "provoked antagonism" described by Schiller in 1927 (205). By this term he means that development of antagonism was observed in minimal conditions or in distilled water. Under starvation conditions, the strains which show no antagonistic effect when sufficient nutrients are present, develop metabolites with whose aid they are capable of utilizing other micro-organisms and whose destructive effect on these micro-organisms can be demonstrated even in a filtrate free from organisms. On the other hand, Bounameaux (29) suggested that the conditions in question give rise to increased lytic antibiotic formation but not to bacteriostasis. However, Savage and coll. (203) have shown that micro-organisms may become habituated to utilizing other micro-organisms as nutrients, but no new antibiotic is produced in this way.

Charrin and coll. (34) showed that the growth-inhibiting effect of pseudomonas on the anthrax bacillus is partly due to

the loss of nutrients, and Manteufel (164), in his study of the inhibitory effect of broth culture filtrates, demonstrated that the inhibition of growth was due to the same cause, "as the inhibitor is thermostabile and filtrable". Barnes (8), too, found no special inhibitory substance in the filtrates, and concluded that a similar reduction in growth can also be produced by diluting fresh culture medium with distilled water. Besta and coll. (16) attributed the inhibition of growth in the so-called staled system exclusively to the loss of nutrients. The same result has been reached by several other investigators, who have regarded nutrient exhaustion either as the exclusive cause (104, 152), or as a partial cause accompanied by loss of energy (38) or by oxidation of the culture medium (126).

Fabry (61) found in 1922 that the urine of a prostatic patient dissolved E.coli although only a white micrococcus could be isolated from it. Culture of this micrococcus with E.coli revealed a phage which could not be demonstrated from the micrococcus or the E.coli alone. The same year, Lisbonne (156) observed that an E.coli strain was capable of producing "phages", but that they had no lytic effect on this strain although they had it on other strains. Thus the phages, too, may in a way be responsible for antagonism.

Kopactzewski and coll. showed in 1923 (144) and again later (4) that the principal cause of antagonism was to be found in physico-chemical changes, such as the changes in pH, ionization, surface tension and viscosity. Other research workers (98), too, have assumed the changes in pH and the loss of O_2 to be at least contributory factors.

In 1924 Bail (7) advanced his mechanical population theory, under which each bacterium requires a certain amount of space in a liquid medium and under identical conditions; hence in a given biological and physical population space each strain finds its maximum concentration, its so-called M-value, which the total of living bacteria cannot exceed. This concentration is usually reached at a temperature of 37° C within 24 hours or sooner. However, Graham-Smith (103) had discovered in 1920 that if the initial concentrations differed much, the maximum concentrations were also different, and in 1926 Cornwall (43) showed that if we inoculate a certain amount of bacteria into

a large volume of fresh culture medium, the maximum concentration will be greater than in a smaller volume of medium. But, if the inoculum is directly proportional to the volume, the ultimate concentration in each is nearly the same. Elaborating Bail's population theory, von Wikullil (230) suggested that a rapidly developing strain will reach its M-value more quickly, and will thus suppress the strains of slower growth. If both strains in a mixed population have the same M-value and rate of growth, the final amount of each strain will be M/2, and the total will equal the M-value of both. Besta and coll. (16) agreed with these findings on the basis of their results obtained from broth cultures. Koblmüller (140) pointed out in his recent publication that, although experience speaks against Bail, the theory evidently holds good but requires additional study.

Vignati (221) claimed in 1926 that antagonism cannot be shown to be due to a single factor but that several factors are always present at the same time, and the whole mechanism is very complex. Many later investigators have arrived at the same conclusion, denying a simple and clear explanation (6, 111), especially for antagonism in vivo (104).

In their investigations into the antagonism between the different strains of one species in 1933, Gundel and coll. (115) found that there must be a certain factor, X, determining which of the strains will win. He called this factor the competitive ability, "Konkurrenzfähigkeit"; it is not connected with virulence or other qualities. A similar conclusion was later reached by Mohr (172), who studied the antagonism in homologous species, e.g. the "staphylococci", S.typhi and other salmonella strains.

As, however, experiments proved that none of the above factors were responsible for the antagonism in every case, Neufeld and Kuhn (177, 178) advanced the concept of "direct antagonism", by which they meant an antagonism of living bacteria manifested directly, without changes in the culture medium. Wynne and coll. studied the phenomenon with bacteria in which no other inhibitors had been found (69, 235), but finally concluded that a highly labile, specific metabolite was involved (236). Gillissen (95) again, claimed that direct antagonism was but one form of adaptation antagonism.

von Mercier (171) showed in 1943 that antagonistic strains

are generally more resistant to external effects such as disinfectants, heat, and defective culture medium. He put forward the suggestion that antagonism proper is not often present; the strains claimed to be antagonistic are in fact better capable of living in poor conditions and therefore survive when the others disappear. But in 1950 Fisher (65) showed that E.coli bacteria when challenged by disinfectants, phages and antagonistic bacteria revealed no difference in the sensitivity to the antagonism of the aerobic, spore-forming bacteria. He admitted, it is true, that the E.coli strains were more resistant when isolated from water because the weaker strains had been eliminated from them.

In his summary, Gillissen (95) listed the following causes of antagonism:

1. Antagonism is caused by constitutional substances normally present, such as:
   (a) antibiotics,
   (b) lipoids and pigments with an antibiotic effect,
   (c) cellulolytic ferments.
2. Antagonism depends on an action which can be defined as adaptation and which presupposes the presence of another organism, the substrate, results in the production of a special, specific substance with an antibiotic effect, may occur in an environment rich or poor in nutrients, and may through mutation, become a permanent quality.

In addition, certain secondary factors, such as changes in pH or rH, loss of nutrients, increase in general metabolites, shortage or surplus of O_2, difference in temperature and growth rate, and difference in M value, may contribute towards the manifestation of antagonism.

ANTAGONISM OF THE GENUS ESCHERICHIA

E.coli antagonism was first noted in 1889, when Lewek (154) found that an E.coli colony disturbed the growth of a neighbouring "Staphylococcus" colony.

Moro and coll. (173) showed in 1906 that the stools of breastfed infants contained factors inhibiting S.typhi and "staphylococci" and, to a lesser extent, E.coli and B.aerogenes, and that E.coli

played an important part in this inhibition. In 1916 Nissle (180) created the basis for the so-called E.coli index determinations when he showed that the various E.coli strains had a varying inhibitory effect on the development of S.typhi. The so-called weak E.coli strains, present, according to him, primarily in various intestinal disorders, did not appreciably affect the growth of S.typhi whereas the strong strains definitely retarded its development. Langer (148) showed that, although the intensity of growth was greater in the strong strains, the rate of reproduction in the weak strains was nevertheless greater. According to him, the strong strains always behaved in the same way, whereas the index figures for the weak strains varied in different tests, though they definitely remained weak throughout. On the other hand, Worpenberg (234) and others later (192) attached no value to the index. According to them, all E.coli bacteria are antagonistic to S.typhi, and no weak E.coli exists.

The antagonism of E.coli to S.typhi is demonstrable only if the inoculum contains plenty of E.coli; if not, S.typhi is antagonistic to E.coli (221). According to Hashimoto, however, this factor depends on the individual variations in E.coli (123), and animal passage adds to the effectiveness of E.coli. According to Tey (213), some E.coli were antagonistic to S.parat.B, while others immediately disappeared.

Antagonism to other E.coli strains can also be shown as parabiosis through a cellophane membrane (228). According to Ørskov (182 a) ordinary E.coli inhibited the dyspepsia coli in special broth, although in ordinary broth the inhibition was hardly observable. Homologous inhibition has been found in filtrates (2) and in staled systems (39). E.coli does not inhibit the Aerobacter in the staled system (39).

Index determinations have also been made with other bacterial strains (141). Similar differences were found in the antagonism of E.coli to "staphylococci" and streptococci. No similar effect was observed on filtrates made of cultures. Index determinations with shigellae (163) also showed differences, although the E.coli strains were more effective against them than against S.typhi. No antibiotics or substances penetrating cellophane were observed in these tests. Reploch (199) found differences in antigenicity as well as in antagonism, the antagonistic E.coli

strains giving higher titre values when tested in the rabbit. The weak strains produced hardly any antibodies at all.

In 1922, van der Reis (198) showed that E.coli produces thermolabile, volatile substances, non-dialyzable and non-filtrable, which inhibit the growth of Corynebacterium diphtheriae. The antagonism of E.coli to C.diphtheriae has also been observed by many other investigators (1, 16). According to Weinstock (226), E.coli did not inhibit the growth of C. diphtheriae, but did inhibit its sugar fermentation. The inhibitory substance was thermostabile.

The antagonism of E.coli to B.anthracis has been studied by many research workers. Gundel (112) demonstrated in 1927 that only living E.coli were antagonistic to it (similarly, 115). According to Rizzi (201) this antagonism and the E.coli -S.typhi antagonism are due to the same factor, although certain differences exist. On the other hand, Giovanardi (98) claimed that the antagonism is only a result of oxygen exhaustion, as practically no antagonism is noted in well aerated broth cultures, and none at all on solid culture medium. It has also been found (99) that in broth the growth of the B.anthracis may later outstrip that of the E.coli.

Pseudomonas strains inhibit E.coli in broth while in ordinary drinking water, on the contrary, E.coli inhibits the Pseudomonas (202).

The antagonism of E.coli to the tubercle bacillus prompted Göttel (102) in 1949 to inject E.coli straight into the tuberculous cavern. The tubercle bacilli disappeared in some cases and the patient's general condition improved. According to Meissner (170), Nissle's potent strains had a greater effect than the weak. Inhibition of the tubercle bacillus was also noted on filtration through a membrane filter. The culture medium was thus regarded as the decisive factor in the origination of the antagonism. According to Hesse and coll. (127), a stable fraction, more fatal to the tubercle bacillus than dihydrostreptomycin though of a weaker static effect, is obtained from broth cultures and the bacterial cells themselves. After phagolysis too (91) E.coli destroyed colonies of tubercle bacillus.

Fungistatic agents were found in E.coli by Wiedling (229), an E.coli infection inhibiting the growth of Penic. notatum.

An alkaline antimycotic acting on yeast fungi was found in E. coli by Thalhammer (214). Paine (183) showed that E.coli controls the occurrence of Monilia and Saccharomyces in the intestine; if E.coli is destroyed by antibiotics, the yeasts grow. Factors inhibiting the growth of yeast were found in the filtrate too (18).

E.freundii has been shown (206) to be antagonistic to Lactobacilli.

In addition to the various forms of antagonism listed above, Gundel (113) reported in 1930 a lipoid fraction with a heterologous inhibitory effect. Fisher (64) showed non-specific inhibitors in the filtrate. Gillissen and coll. (97) demonstrated adaptation-antagonistic factors restraining the development of S.typhi in mixed culture filtrates of S.typhi and E.coli. McLeod and coll. (168) showed in 1921 that thermolabile substances, autoinhibitory and Shigellae-inhibiting, developed in the filtrate of well aerated systems.

Colicines. — Gratia (105) showed in 1925 that an E.coli strain which was virulent for guinea-pigs and rabbits produced a factor lytic to another E.coli strain; however, it was not a phage. The factor was diffused through solid culture medium, dialyzable and thermostabile for half an hour at 120° C, resisted chloroform, and failed to precipitate with calcium triphosphate. Guelin (111) in 1943 found a thermostabile, specific non-transmissible lytic substance, which required Ca for its formation, and was neither phage nor enzyme. Zamenhof (238) found a strain which produced a substance lytic to only one of the E.coli strains tested, and which did not affect gram-positive bacteria either. The substance was thermostabile, and had no static effect. Heatley and Florey (125) reported in 1946 on a technique by which colicines could be isolated. Production was greater in well aerated systems. In addition, many E.coli strains developed hydrogen peroxide on a glucose-containing medium; this must not be confused with colicines proper. Fredericq and coll. (83) showed that the colicine effect was not similar to the antagonism obtained with the staled system. In his very comprehensive monograph Fredericq (75) showed that colicines were antibiotics

developed by various enterobacteria with a specific effect on other enterobacteria.

Greatly varying reports on the occurrence of colicines are found in the literature. According to Halbert (119), 10 per cent of 1,234 strains studied were strongly and 8.3 per cent weakly colicine-producing, while 81.7 per cent produced no colicine when tested with Flexner III strain. According to Blackford and coll. (21), up to 79.2 per cent of the E.coli strains produced colicines. Mainly E.coli, and to some extent E.freundii produce colicines but the Sigellae and the Salmonellae very seldom (79). According to Levine and coll. (153), only 19 per cent of the E.coli found in stools and water, but 69.2 per cent of the E.coli isolated from the nasopharynx of children produced colicines.

The production of colicines in vitro is best on a thin agar layer; in broth the production is almost nil (120). Production is increased by ultraviolet irradiation and by mutagenic and cancerogenic substances but not by sensitive strains (131). Glucose in the medium is disadvantageous as it changes the pH, for colicines are not produced under pH 5.5 and over pH 10.5 (21). On the other hand, Beumer (17) found that a lysogenic strain produced phages in a neutral environment, colicines in an alkaline, but neither in acid. When lyophilized colicine strains are awakened, production starts immediately (181). If the parent strain mutates, the variation may produce different colicines with unchanged spectrum and resistance qualities but changed antigenicity and thermoresistance (25). When strains producing colicine in vivo are injected intraperitoneally into the experimental animal, colicines will be found in the serum and peritoneal exudate (121).

The colicines have a lytic effect. Unlike other antibiotics, their inhibition zone on agar does not appreciably change on 10-fold dilution (120). Evidently in this case colicines are plentiful, for small amounts can be titrated with sensitive bacteria (131); the effect is reminiscent of the toxin-antitoxin reaction, in the form of a fast reaction similar to adsorption, and given amounts corresponding to one another (78). The effect is mainly is-antibiotic, affecting related strains, but not auto-antibiotic, affecting the strains itself (71). The Escherichiae, and Shigellae are often sensitive, the Salmonella, Aerobacter and Proteus

groups seldom (79). Only one of the known colicines, H, has an action on Proteus species (71). A total of 17 different colicines are known; they differ in cross-resistance, thermostability, dialysis, sensitivity to proteases and the form of the inhibition zone (75, 79). Some strains can produce several colicines, and several colicines may have a lytic effect on the same strain. A sensitive strain has so-called receptors on or through which the effect of colicine occurs. The colicines are polypeptides in character (79). In contrast to Fredericq, Cook and coll. (42) found that the streptococci, pneumococcus, C.diphtheriae, Mycob. smegmae and the Neisseriae are sensitive to some colicines. They also consider some staphylococcal strains as slightly sensitive, although Halbert (119) claims that the Staphylococcus is resistant.

It has been possible to show electrophoretically that some of the colicines possess a negative, others a positive charge (161).

Spontaneous mutations of sensitive strains result in resistant strains (73), in which, however, only the receptor of the colicine in question becomes resistant, the other receptors remaining free, and the strain being sensitive to the effect of other colicines (74). A resistant strain does not differ from the parent strain morphologically, in antigen properties, or in biochemical properties (74).

Proteus and the enterococci destroy colicines (80); anti-serum prepared on a homologous strain, (26) or an extract of the sensitive strains (27) inhibits the effect of colicine by blocking the receptors, in the same way as phages.

The antigen structure and colicine production have nothing in common (109, 161), nor have the biochemical reactions, except that E. freundii produces colicine A and the "paracoli" strains colicine J and K (72).

Colicines and phages have many properties in common (132). They have common receptors (82) and hence cross-resistance. On a solid medium, colicine may form plaques simulating phages (77). Lysogenic strains too may produce colicines (17, 76).

ANTAGONISM OF THE GENUS MICROCOCCUS

As early as 1888 de Freudenreich (84) found that a filtrate made of M.pyogenes v. aureus cultures inhibited the development

of the causative agents of glanders and chicken cholera completely, that of S.typhi markedly, and that of M.tetragenus moderately. A filtrate of M. pyog. v. albus cultures completely inhibited the development of S.typhi and the causative agent of chicken cholera. In 1897 Ghadially (92) reported that Microc. Ghadiallii were capable of destroying intestinal bacteria. Krencker (146) reported in his doctorial thesis in 1903, that 4—6 week old "staphylococcus" cultures dissolved the anthrax bacillus. In his studies of the causative agents of urinary tract infections Faltin (62) demonstrated in 1908 that when growing in the urine the "staphylococcus" can kill E.coli. An alcohol extract made of "staphylococcus" has been found to have a bactericidal effect on the Bac. anthracis (184) and on other bacteria as well (113).

As regards the antagonism of micrococci to the diphtheria bacillus, de Witt (232) showed in 1912 that in vivo they contributed to the disappearance of diphtheria but were ineffective in vitro. Gate and coll. (90) showed that "staphylococci" did not inhibit avirulent strains in vivo or in vitro, but in 1925 Adachi (1) found antagonism in vitro, as did Besta and coll. (16) in broth cultures. According to Duliscouet and coll. (55) some of the strains inhibited, while others increased the growth of pathogenic diphtheria bacilli (53); however, he considered that indifferent strains also existed. An inhibitory strain was found to have a lytic effect specific for Corynebacteria. The agent in question is thermolabile, and non-filtrable, and its effect is enhanced by calcium (54). Lesbre and coll. (151) found a viscous form of micrococcus which has an inhibitory effect on diphtheria bacillus in vivo also.

Gratia and coll. (108) reported in 1924 that living micrococci digested killed homologous strains. Fleming (67) showed in 1932 that M.pyogenes v. aureus inhibits the development of Serratia. According to Mohr (172), M.pyogenes v.aureus inhibits the growth of M.albus, which in turn inhibits the growth of M. citreus. According to Joyeux and coll. (135), the micrococcus inhibits the growth of Microsporon felineum at a temperature of 37° C but not at room temperature. In 1943 Hutchinson and coll. (130) isolated from water a micrococcus inhibiting the growth of E. coli.

Jennings and coll. (134) showed that 23 out of 205 nasopharyngeal micrococci were active against Corynebact. xerosis, and several of the strains against other micrococci and the diphtheria bacillus. Both M. pyogenes v.aureus and M.pyog. v. albus showed active strains. Kelly and coll. (137) showed that the alcohol precipitate of the aqueous extract of "staphylococci" inhibits the growth of some Gram-negative rods. In a medium containing urine or urea, Microc. ureae produces enough NH_3 to inhibit the growth of other bacteria (129), above all of S. typhi and the Shigellae. Ritter (200) remarked in 1954 that, on the whole, micrococcal antagonism had been little studied. From one strain he was able to isolate a thermostabile substance, similar to an antibiotic, inhibiting the growth of Lactobac. Helveticus, L. lactis and L.thermophilus. Prica (190) had shown previously that a thermostabile, filtrate substance passing through a collodion membrane and inhibiting the nasopharyngeal Klebsiella strains was obtainable from "staphylococci". This substance had both a bacteriostatic and bactericidal effect. Dujardin -Beaumetz (50) showed in 1932 — but only on plate — that a staphylococcal strain inhibited almost all the Gram-positive mycobacteria and Nocardiae, including cold-blooded bacilli; it may have been a micrococcal strain which produces hydrogen peroxide. In addition, he found (51) a saprophytic micrococcus which inhibited the growth of almost all bacteria except Proteus. Benetti and coll. (24) showed that very often hydrogen peroxide is the inhibitor in micrococci. In 1948 Su (211) isolated from micrococcus an antibiotic, the micrococcine, effective on Gram-positive bacteria. Usually hydrogen peroxide is obtained from micrococci but the "staphylococci" are the real antibiotic producers (Su 210). In 1949 Gardner (87) isolated a substance precipitable by trichloro-acetic acid, which was thermoresistant when neutral and acid. Loeb (159) reported four types of antibiotics in micrococci; they affected the Gram-positive, but not the Gram-negative bacteria, and were polypeptides in character. He isolated a polypeptide with antibiotic effect from Micrococcus epidermis (160). Murray and coll. (175), continuing the work, reported that the active antibiotic isolated from M.epidermis was effective on Gram-positive bacteria. The four types of antibiotics differed in dialyzability, stability,

production curve, and spectre; all had a bacteriostatic effect. Only producers of hydrogen peroxide are found among air-borne micrococci (12, 150).

Fredericq and coll. (81) and Halbert and coll. (122) showed that the "staphylococci" have a system analogous to that of enterobacteria. There are several staphylococcines, corresponding to colicines, which have their specific receptors in the sensitive strains; they differ in precipitation, thermostability, dialyzability, and cross-resistance. Many of them are polypeptides. The inhibitory effect extends also to streptococci, clostridia and mycobacteria. The Gram-negative bacteria are completely resistant to them, as are the Candidae.

ON ANTAGONISM BETWEEN THE GENERA ESCHERICHIA AND MICROCOCCUS

Krogius and Wallgren (147) found in 1899 that when "staphylococci" and E.coli are grown in mixed cultures on urine, the latter usually lags behind in development; in some cases, however, the two were equal, or E.coli could develop better. The "white staphylococcus" was weaker in their experiments. Conradi and coll. (41) reported in 1905 that in mixed cultures with E.coli all bacteria but the "staphylococci" succumbed. Konrad (143) discovered in 1908 that E.coli and the "staphylococci" often lived in symbiosis in mixed cultures. In the same year Faltin (62, 63) found that if E.coli and the "staphylococci" are grown in mixed cultures on urine in tubes, the latter will prevail the more easily, the greater the difference in its favour in the amounts inoculated. If, again, the amount of E.coli inoculated is greater, E.coli may restrain the development of the "staphylococcus" or even kill it, if the superiority is large enough. On the other hand, in an artificial bladder where the urine used as culture medium was changed at intervals, E.coli always killed the "staphylococcus". According to Faltin, E.coli produced metabolites fatal to "staphylococci". These substances are filtrable through a Chamberland filter, are absorbed to carbon and are thermolabile, boiling reducing their effect.

With mixed cultures grown on broth, Gundel and coll. (114) found that after 24 hours the "staphylococci" were no longer culturable from among the E.coli. Similar results were attained by von Wikullin (230) and Gillissen (94). A filtrate of E.coli culture has been found to possess a definite inhibitory effect on the growth of Microc.p.aur. (18). On the other hand, Gillissen and coll. noticed no adaption antagonism proper (97), although he later claimed that some activity occurred on mixed culture filtrate (94). Regnier and coll. (197) counted the total of bacteria in mixed cultures and found that "staphylococcus" did not affect the growth of E.coli but that *after* the logarithmic phase E.coli destroyed "staphylococcus" the more effectively, the greater the superiority of E.coli in the primary inoculation.

von Eisler (57) found in 1918 that E.coli killed with heat had an inhibitory effect on "staphylococcal" growth in so-called staled agar. According to McLeod and coll. (168), a pour-plate formed of E.coli, covered by a thin sterile layer of agar, partially inhibited "staphylococcal" growth; however, a corresponding "staphylococcus" plate did not inhibit E.coli. According to Lockhard and coll. (157) Micrococcus pyogenes did not grow on staled agar made with E.coli, and staled agar made with the micrococcus did not inhibit E.coli.

Koch and coll. (141) found by E.coli index determinations according to Nissle that the different E.coli strains had a different effect, and that the different "staphylococci" were varyingly sensitive to E.coli. It has also been found that if an E.coli strain is strongly antagonistic to S. typhi it is also strongly antagonistic to "staphylococcus".

Grassi (104) claimed that the inhibitory effect was due only to loss of a nutrient medium. In their parabiosis experiments with twin tubes divided by a dialysis membrane, Wheeler and coll. (228) found that E.coli inhibited "staphylococcus". Goldsworthy and coll. (100) found that E.coli colony excreted a substance diffusible through agar and inhibitory to Microc. lysodeicticus.

Generally, investigators have held the view that the colicines have a specific effect on enterobacteria and that the effect does not extend to the "staphylococci" (25, 75, 88, 119). But Cook and Blackford and coll. (20, 42) found that 4 out of 47 E.coli

inhibited "Staphyloc.aureus", e.g. type strain FDA was slightly sensitive to the colicines. On the other hand, the antibiotic produced by "staphylococci" has been found to have some effect on E.coli (87).

Lavergne and coll. (149) claimed in a report published quite recently that the antagonism of E.coli to Micrococcus is due only to "direct antagonism", as their experiments revealed no other factors.

The contradictory information found in the literature on the relations between the genera Escherichia and Micrococcus, usually based on experiments with a few strains, the results of which are generalized and on the observation that these strains are often simultaneously present in normal and pathological flora, has occasioned the present investigation, which represents an attempt to answer the following questions:

1. Are all the Escherichia strains antagonistic to the micrococci? And are all the micrococci equally sensitive to the antagonistic effect of the Escherichiae; if not, does the sensitivity show any correlation with the various biochemical reactions and/or the general sensitivity to antibiotics or with the possible occurrence of micrococci separately or mixed with Escherichia strains when isolated?

2. With strains selected from a mass investigation, can any of the previously proposed causes of antagonism be shown to be the factor principally responsible, or can such a possibility be completely excluded for any of the causes?

II PRESENT INVESTIGATION

1

ANTAGONISM AMONG ESCHERICHIA AND MICROCOCCUS STRAINS COLLECTED AT RANDOM

In order to obtain as comprehensive a picture as possible of the extent and strength of the antagonism of the Escherichia strains, on the one hand, and of the sensitivity of micrococci to this antagonism, on the other, and to discover possible correlations with the micrococcal activity or the isolation environment, 100 different Escherichia strains and 150 different micrococcal strains were collected and the antagonism studied by two different techniques.

MATERIAL

Escherichia Strains. — All the strains were motile, Gram-negative rods, which fermented glucose, mannitol and lactose, forming acids and gases within a maximum of 48 hours, did not dissolve gelatins, gave a negative Voges-Proskauer reaction (with the exception of Strain A_{46}, which was positive) and did not decompose urea. By indole formation, H_2S formation and growth with ammonium citrate as the only source of carbohydrates, the strains could be classified into the sub-groups [1]) listed in table 1; the atypical intermediate forms are here called the intermediate strains.

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[1]) All determinations and classifications of the strains were effected according to the instructions given by Kauffmann (136) wherever applicable. All reactions which differed from those expected were checked by re-determinations. Negative reactions were followed up for 7 days if necessary.

TABLE I

Escherichia strains

Type	Reaction	Number	Strain No.
E. coli	Indole + H_2S — Amm. citr.—	80	II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII, XIX, XX, XXI, XXII, XXIV, XXV, XXVI, XXVIII, XXX, XXXI, XXXII, XXXIII, A_1, A_3, A_4, A_5, A_6, A_8, A_{10}, A_{11}, A_{12}, A_{13}, A_{14}, A_{15}, A_{16}, A_{17}, A_{18}, A_{19}, A_{21}, A_{22}, A_{24}, A_{25}, A_{26}, A_{27}, A_{28}, A_{29}, A_{31}, A_{32}, A_{33}, A_{34}, A_{35}, A_{36}, A_{37}, A_{38}, A_{39}, A_{40}, A_{41}, A_{45}, A_{47}, A_{48}, A_{49}, B_6, B_8, C_1, D_1, D_5, D_6, D_8, D_{10}, D_{15}, D_{16}, D_{18}, D_{19}
	Indole — H_2S — Amm. citr. —	1	XXVII
	Indole + H_2S — Amm. citr. +	4	I, XXIII, A_2, B_3
Intermediate strains	Indole — H_2S — Amm. citr. +	3	A_7, A_{44}, B_1
	Indole + H_2S + Amm. citr. —	2	D_{13}, D_{17}
	Indole — H_2S + Amm. citr. —	1	A_{30}
	Indole + H_2S + Amm. citr. +	4	XVIII, A_{43}, A_{46}, B_2
E. freundii	Indole — H_2S + Amm. citr. +	5	XXIX, A_9, A_{20}, B_4, D_3

E.coli strain XV, which was typical biochemically, differed from the others in that it was unable to grow on a peptone-free culture medium, (blood agar used in sensitivity determinations), although its growth on a simple peptone-containing medium was normal compared with the others.

Eight of the Escherichia strains (X, C and B strains) were isolated from various pus specimens, 14 (I-IX and XI-XV) from urine, 31 (XVI-XXXIII and Strains D) from stools of healthy adults and the 47 A Strains from the stools of children with diarrhoea. The strains were kept on agar slants in tubes containing ordinary nutrient agar, and the cultures were made as

mass inoculations thus avoiding the mutations which might have been present in individual colonies. The agar slant cultures were renewed at intervals of approx. two months or more frequently, and were kept in the meantime in a refrigerator at a temperature of + 4 — + 10° C.

Micrococcus Strains. — All the strains were catalase-positive, Gram-positive cocci, occurring by pairs or in irregular clusters, and none of them were able to grow with ammonium as the only source of nitrogen, or to decompose starch (185). 109 of the strains were coagulase-positive Micrococcus pyogenes v. aureus producing a yellowish pigment, 5 coagulase-positive M.pyog.v.albus producing a white pigment, 4 M.candidus, 1 M. candidus mannitol-positive, 2 M.albus apathogenes, 16 M.epidermidis (of which 8 were atypical), 9 M.epidermis not dissolving gelatin, and 4 unidentified micrococci (185).

Eleven of the micrococcal strains were type strains (Oeding's serological strains 17 A, 2253, 365 S, 1503, 28, F 21, 2095, 3647, 3189; Type Strain Oxford and Type Strain 209 P), 44 had been isolated from the stools of children with diarrhoea (Strains A), 6 from the stools of healthy adults (86-91), 14 from the nasophrynx of healthy adults (Strains D), and the remaining 75 from various specimens submitted for routine tests (urine 20, pyodermie 12, pus 6, cutaneous excretions 26, eye excretion 1, ear excretions 5, expectorations 2, and the source was not known of 3). The faecal and nasopharyngeal strains were isolated by means of blood agar containing potassium chromate (185), the rest by means of ordinary blood agar. The strains were stored in the same way as the Escherichia strains.

Culture Medium. — Unless otherwise specified, the following peptone broth was employed in all the experiments:

Proteose peptone No. 3 (Difco)	20.0
Glucose	0.5
NaCl	5.0
Na_2HPO_4	5.0
H_2O, distilled	1000.0

Sterilized by autoclave at 120°C, pH 7.4.

For solid media, unless otherwise specified, 2 per cent of agar was added to this basic culture medium.

TECHNIQUES

Staled Agar Technique. — The antagonistic strain studied was cultured from agar slants on 2 ml of peptone broth and incubated at 37° C overnight, after which a loopful was transferred into a 200 ml bottle of peptone broth. The bottles were incubated for 24 hours at 37° C in a rotator in which the bottles revolved at an approximate distance of 15 cm from the central axis at an angle of 45° and at a speed of approx. 50 RPM. Subsequently, equal amounts of the culture and of 4 per cent peptone agar, melted and cooled down to 50° C, were mixed and poured onto 150 mm. diameter plates. After setting, the plates were dried for half an hour at 37° C and inoculated as a

streak culture in the form of a zigzag line with the strains of the other genus to be tested. They were grown overnight on 2 ml of peptone broth, and then transferred by loop into a tube containing a fresh 2 ml of peptone broth, which was incubated for 4 hours and from which the inoculation onto staled plates took place. On each plate 25 strains to be tested were inoculated radially. At the same time a 2 per cent peptone agar plate of the strains tested was inoculated to serve as a growth control, with which the colonies, which might develop on the staled plates, could be compared. The plates were incubated for 48 hours at 37° C and the results were determined macroscopically, the intensity of growth being defined in terms of colony size.

Antibiotic Technique. — The strain (Micrococcus) to be tested was grown in a 2 ml peptone broth tube overnight. The culture was diluted 1:10,000 (or less if the growth appeared weak macroscopically) with sterile saline solution, and an even culture was spread by a sterile cotton pad on a 150 mm diameter plate of 2 per cent peptone agar. The plates were dried for half an hour at 37° C, after which the strains (Escherichia) to be tested were inoculated as stab cultures from a 24-hour agar culture onto the plate, 25 of the Escherichia strains to be studied onto each plate. The plates were incubated for 24 hours at 37° C, and any inhibition of growth around the antagonistic colonies was recorded by the naked eye (Photo 1).

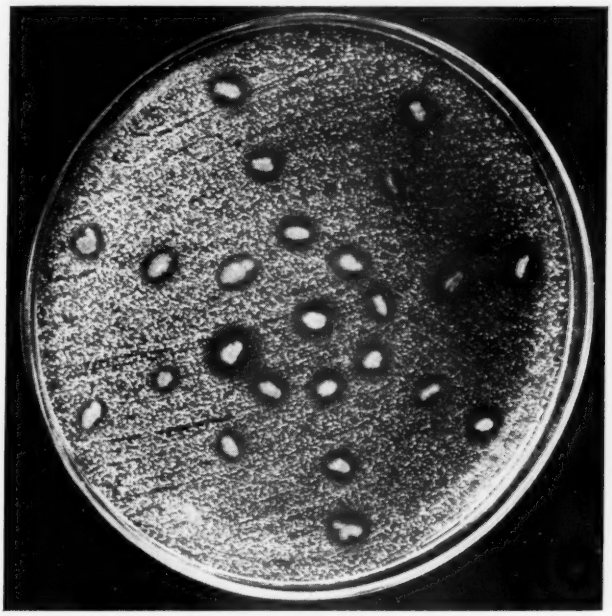


Photo 1. Zones of inhibition caused by Escherichia on a sensitive micrococcal strain.

Note the completely inactive colony of Escherichia at the bottom of the picture.

MATHEMATICAL FORMULAE

The following mathematical formulae have been employed in the treatment of the material:

1. The index of the antagonistic strain with the staled agar method:

$$X^E = \frac{an - \Sigma i^E}{an}$$

a = maximum value of colony size on staled plate
n = number of test strains
i = size of colony verified
Σi^E = sum of the colony sizes of test strains grown with one antagonistic strain.

2. Value of the resistance of the test strain with the staled agar method:

$$X^M = \frac{\Sigma i^M}{an}$$

Σi^M = sum of the colony sizes of test strains grown with the different antagonistic strains.

3. Index of the antibiotic strain with the antibiotic method:

$$Y^E = \frac{\Sigma e^E}{bn}$$

b = maximum value of the size of the inhibition zone
e = verified size value of the inhibition zone
Σe^E = sum of the inhibition zone values obtained by the different test strains with one antagonistic strain.

4. Resistance of the test strains to antibiosis with the antibiotic method:

$$Y^M = \frac{bn - \Sigma e^M}{bn}$$

Σe^M = sum of the inhibition zone values produced by the different antagonistic strains with one test strain.

5. The formulae employed in the statistical treatment:

Observation series $x_1 \ldots\ldots\ldots\ldots x_n$. Arithmetic mean $\bar{x}$
Dispersion of a single observation

$$s = \sqrt{\frac{\Sigma (x_1 - \bar{x})^2}{n - 1}}$$

Observation series $x_1 \ldots\ldots\ldots\ldots x_n$ and $y_1 \ldots\ldots\ldots y_n$. Means $\bar{x}$ and $\bar{y}$, dispersions s_1 and s_2. The difference of the means, including dispersion, is given in the following form:

$$\bar{x} - \bar{y} = \sqrt{\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 n_2}}$$

PRELIMINARY TESTS OF THE STALED AGAR TECHNIQUE

The following series of tests was made to determine the optimal incubation period and the primary culture method for the strains of both Escherichia and Micrococcus, which would produce the maximum antagonistic effect with the staled agar technique. E.coli strain XIII, selected at random, was inoculated into 10 Erlenmeyer flasks, of which five were grown at 37° C in a rotator and the remaining five at 37° C standing on an incubator shelf. After incubation periods of 6 hrs, 24 hrs, 48 hrs, 72 hrs and 120 hrs the cultures were mixed with 4 per cent peptone agar melted and cooled to 50° C and the plates were poured. Seven different micrococcal strains from both the 24-hour and 4-hour peptone broth cultures were inoculated into them.

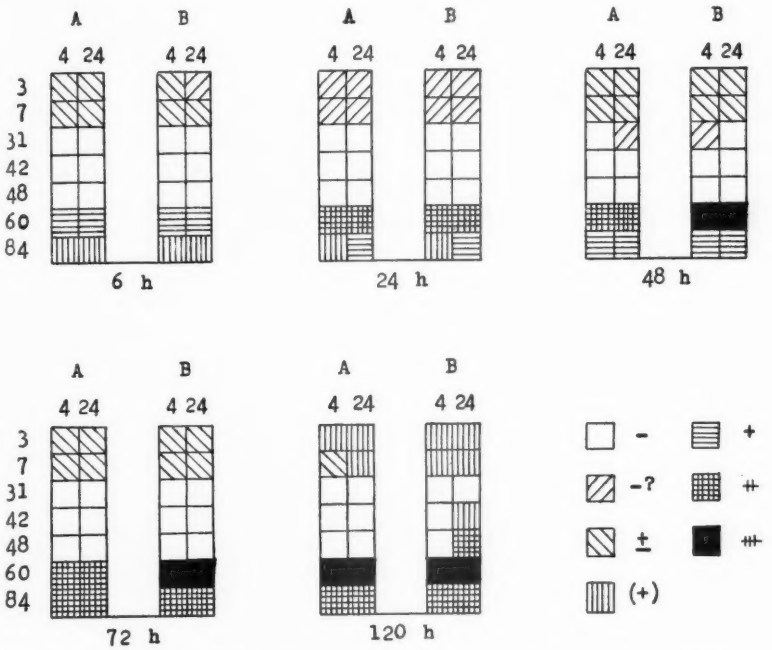


Fig. 1. Diagram showing the antagonistic effect of E.coli strain XIII on seven micrococcal strains on staled agar.
A = plates made of culture grown at rest
B = plates made of culture grown in the rotator.
4 and 24 = primary incubation periods of the micrococcal strains.
—, —? etc. = verified size of micrococcal colonies, detailed description in the text.
Figures at the left indicate the micrococcal strains.

The results are shown in Fig. 1. The 6-hour Escherichia cultures were already found to have an inhibiting effect on the micrococci and this effect was even more marked than that on the plates made from 120-hour cultures. The effect seemed to be at its maximum on plates made from the 24-hour cultures and the Escherichiae grown in the rotator obviously had a slightly stronger antagonistic effect, manifested particularly in the inhibition of Micrococcus strain 60 on plates made from 48-hour and 72-hour primary incubation cultures. On the other hand, fresh micrococcal culture growing in the logarithmic phase seemed capable of greater resistance to the antagonistic effect than a 24-hour culture in the stationary phase.

In order to determine the stability of the staled agar technique, repeated determinations were made with two E.coli strains and 16 microccal strains, as follows. For E.coli I, plates (dia. 9 cm) were made of two cultures, (A) 8 and (B) 16 micrococci strains being inoculated into each, all in the form of a zigzag line. Similarly, for E.coli A_{34}, plates were made of two cultures and micrococci inoculated as above. The results, given in Table 2 omit micrococcal strain A_{22} which failed to grow on any of the plates of these test series or on the control plate.

TABLE 2

Stability controls of the staled agar method

Micrococcus No	I — Escherichia coli I Made on two cultures							
3	2	2	2	2	2	2	2	2
7	3	3	3	3	2	2	2	2
57	3	3	3	3	3	3	3	3
63	3	3	3	3	3	3	3	3
68	0	0	0	0	0	0	0	0
88	1	0	0	0	1	1	1	1
A_1	3	3	3	3	3	3	3	3
A_8	2	2	2	2	2	2	2	2
A_{15}	1	1	1	1	1	1	1	2
A_{19}	2	2	2	2	2	1	2	2
A_{34}	2	2	3	3	3	2	2	3
A_{48}	3	3	3	3	3	3	3	3
B_6	1	1	2	2	2	2	2	2
D_3	0	0	0	0	1	1	1	1
D_8	0	0	0	0	0	0	0	0
	42.4	44.0	40.0	40.0	37.8	42.2	40.0	35.6

M = 40.2 s = 2.6

TABLE 2, cont'd

II — Escherichia coli A_{34}

Made on two cultures

Micrococcus No								
3	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1
57	3	3	3	3	2	2	2	2
63	3	3	3	3	3	3	3	3
68	0	0	0	0	0	0	0	0
88	0	0	0	0	0	0	0	0
A_1	3	3	3	3	2	3	3	3
A_8	0	0	0	0	1	1	1	1
A_{15}	0	0	1	1	0	1	1	1
A_{19}	1	1	1	1	1	1	1	1
A_{34}	0	0	0	0	0	0	0	0
A_{48}	3	3	3	3	3	3	3	3
B_6	0	1	0	1	1	1	1	1
D_3	0	0	0	0	0	0	0	0
D_8	0	0	0	0	0	0	0	0
	66.7	64.4	64.4	62.2	66.7	62.2	62.2	62.2

M = 63.9 s = 1.99

total s = 2.3

From the results obtained, the statistical dispersion was calculated to be 2.3. Hence the technique can be considered sufficiently stable to justify the drawing on the basis of colony size of conclusions regarding the intensity of the antagonism and the resistance of test organisms.

GROWTH OF MICROCOCCI ON STALED AGAR PLATES MADE FROM ESCHERICHIA STRAINS

The growth of 150 micrococcal strains on staled agars made on 100 Escherichia strains was studied. Before pouring the plates the acidity of the Escherichia cultures was determined by indicator paper. In all strains it remained within the range of pH 7—7.5.

The sensitivity of the micrococcal strains and at the same time the antagonism of the Escherichia strains was estimated from the size of the micrococcal colonies. Micrococcal colonies hardly discernible to the eye at the beginning of the inoculation line were classified under complete inhibition, as inoculation may leave on the agar surface a trace easily mistaken for a very

weak growth. Pinpoint colonies along the whole inoculation line were listed as very weak growths. Distinctly pigmented colonies, average size 0.3 mm, were listed as weak growths. Colonies of approx. 0.5 mm were classified as moderate growth. Colonies slightly smaller than those on the control plate were listed as good growths and those equal in size to the colonies on the control plate as excellent growths. For micrococcal strain 60, the growth was often found to exceed that on the control plate whereas for the other strains the growth never exceeded that on the control plate.

Antagonistic Intensity of Escherichia Strains. — The antagonistic intensities of the Escherichia strains, estimated from the size of the micrococcal colonies, are given in Table 1. The following values were used to indicate the antagonistic intensity: Complete inhibition was indicated by 0, weak growth by 1, moderate growth by 2, and good or exellent growth by 3. The values for the different micrococci grown on the staled agars of each Escherichia strain were added up, and the final

TABLE 3

Antagonistic intensity of Escherichia strains on staled agar, computed according to Formula I

Value	E. coli	Intermediate	E. freundii	Total
98	2			2
97	1	2	2	5
96	11	3	1	15
95	19	1	1	21
94	15	1	1	17
93	12			12
92	6	1		7
91	4			4
90	2			2
89	3			3
88	2			2
87	1	1		2
86	3			3
85	1			1
84				—
83	2			2
67	1	1		2
	85	10	5	100

antagonistic index, independent of the number of strains, was computed from this sum according to Formula I. The final index is proportional to the inhibitory effect of each Escherichia strain.

Table 3 shows that the Escherichia strains generally are strongly antagonistic (possible maximum = 100) but that minor individual variations exist. Definitely weak Escherichia strains number 15; two of them, Strains XV and D_{17}, are remarkably weak. Remarkably strong strains total 7, of which strains A_{25} and A_{27} are the strongest. Both of them are strains isolated from diarrhoeal stools, serological type Dc canioni.[1]) However, none of the Escherichia strains completely inhibited the growth of all micrococcal strains. The table shows, further, that although the antagonism cannot be shown to depend on any individual biochemical factor, the few Intermediate and E.freundii strains in the material are obviously more numerously represented among the strains classified as strongly antagonistic.

Resistance of Micrococcal Strains. — The value (0—100) given to each micrococcal strain, indicating the resistance of the micrococcus, was computed according to Formula II. The resistance values of the micrococcal strains are given in Table 4.

The table shows that although some 90 per cent of the micrococcal strains seem to be relatively sensitive to the antagonistic effect of Escherichia, there are among them three exceedingly resistant strains: 60, value 93, 63, value 91, and A_{48}, value 79. Ten strains failed to grow on staled agars made of any of the Escherichia strains.

In addition, it will be seen that all the strains of Micrococcus pyogenes v. aureus are relatively sensitive, while both the most sensitive and the most resistant strains are found among the apathogenic micrococci. The few M.candidus strains of the material are among the most sensitive. However, no absolute correlation can be demonstrated with any individual biochemical reaction, nor with the colour of the pigment, the general sensitivity to antibiotics [which for these strains was determined in connection with an earlier investigation, (186)] nor with the

[1]) Only 7 of the Escherichia strains were serologically determined by slide agglutination at the State Serum Institute.

normal colony size. Table 5 shows that micrococcal sensitivity to the antagonism of Escherichia on staled agar is not affected by whether or not the micrococci were isolated as mixed populations with Escherichia.

TABLE 4

Resistance of micrococcal strains to the antagonistic effect of Escherichia on staled agar, computed according to Formula II

Value	Microc. pyog. v. aur.	Microc. pyog. v. alb.	Microc. cand. mann —	Microc. cand. mann +	Microc. epid. gel +	Microc. epid. gel —	M. albus apath.	Unid.	Total
93					1				1
91								1	1
79								1	1
51					1				1
43		1							1
36							1		1
34	1								1
33	1								1
31	1								1
24					1	1			2
23					1				1
21					1				1
20					1				1
19					1				1
16		1							1
15	1								1
14	1								1
11	2					1	1		4
9	3					1			4
8	3								3
7	2								2
6	2								2
5	3								3
4	11	1							12
3	12								12
2	21					2			23
1	32	2			1	1		1	37
Under 1	12		3		3	1		1	20
0	1		1	1	5	2			10
	109	5	4	1	16	9	2	4	150

TABLE 5

Comparison of the resistances on staled agar of micrococci isolated from the same samples as Escherichia and from elsewhere

Value	Isolated together with Escherichia		Isolated separately from Escherichia	
	All	M.pyog.v.aur.	All	M.pyog.v.aur.
93	—	—	1	—
91	—	—	1	—
79	1	—	—	—
51	—	—	1	—
43	1	—	—	—
36	1	—	—	—
34	—	—	1	1
33	—	—	1	1
31	—	—	1	1
24	1	—	1	—
23	—	—	1	—
21	1	—	—	—
20	—	—	1	—
19	—	—	1	—
16	1	—	—	—
15	—	—	1	1
14	1	1	—	—
11	1	1	3	1
9	1	—	3	3
8	1	1	2	2
7	1	1	1	1
6	—	—	2	2
5	1	1	2	2
4	4	4	8	7
3	3	3	9	9
2	8	7	15	14
1	20	19	17	13
Under 1	8	8	12	4
0	—	—	10	1
	55	46	95	63
	M = 6.1	M = 2.3	M = 6.9	M = 4.7

All ..... difference 1 ± 2.4
M.pyog.v.aur. .... difference 2.3 ± 1.2

ANTIBIOTIC EFFECT OF ESCHERICHIA STRAINS ON MICROCOCCI

The growth inhibition caused by stab cultures of 100 Escherichia strains on 150 micrococcal strains on agar was studied. The antibiotic effect was determined according to the diameter of the inhibition zone produced by the Escherichia colonies. What looked like a reduced growth immediately around the Escherichia colony was found very frequently and was omitted in the calculations. A true inhibition zone only was taken to indicate an antibiotic effect. An inhibition zone of 1 mm was classified as weak inhibition, one of 2 mm as moderate inhibition, one of 3 mm as good and one of 4 mm or more as excellent inhibition. The estimations were made macroscopically (Photo 1).

Antibiotic Effect of Escherichia Strains. — The antibiotic value was computed from the size of the inhibition zones as follows: no inhibition, value 0, weak inhibition, 1, moderate, 2, good, 3, and excellent inhibition, 4. The inhibition values obtained for each micrococcal strain were added up, and the sum, computed according to Formula III, gives the antibiotic antagonistic index of the Escherichia, which is directly proportional to the intensity of the antagonism. The results are given in Table 6.

The table shows that the bulk of the Escherichia strains cannot be taken as producing antibiotics effective on micrococci. However, some 10 per cent of the strains had a definite antibiotic effect, the strongest among them being Strains A_{19}, index 38, and A_{49}, index 31. Six strains, I, XXVI, XXVII, XXIX, A_{22} and B_2 showed no effect on any of the micrococci. In addition, it will be seen from the table that, even though the antibiotic production cannot be considered to depend on any individual biochemical reaction, the few Intermediate and E.freundii strains in the material seem to be generally weak antibiotic-producers.

TABLE 6

Antibiotic intensity of Escherichia strains, computed according to Formula III

Index	E. coli	Intermediate	E. freundii	Total
38	1			1
31	1			1
30	1			1
28	1			1
23	1			1
21	1			1
20	2			2
12	1			1
10	1			1
7	3	1		4
6	6			6
5	4	2		6
4	8			8
3	11	1	1	13
2	15	2		17
1	13	1	1	15
Under 1	11	2	2	15
0	4	1	1	6
	85	10	5	100

Resistance of Micrococcal Strains. — Micrococcal resistance was computed according to Formula IV from the sum of the values obtained from the size of the inhibition zone and indicates in direct proportion the resistance of the micrococci to the antibiotic effect of the Escherichia. The results are given in Table 7.

TABLE 7

Resistance of micrococcal strains to the antibiotic effect of Escherichia, computed according to Formula IV

Value	Microc. pyog. v. aur.	Microc. pyog. v. alb.	Microc. cand. mann —	Microc. cand. mann +	Microc. epid. gel +	Microc. epid. gel —	M. albus apath.	Unid.	Total
100	9	1	1					1	12
99	37	1						1	39
98	19	1		1	1		1		23
97	18		1						19
96	12				1	1			14
95	4		1			1			6
94	3					2			5
93	6	1			1	1			9
92	1					1			2
91					1	2			3
90		1			2				3
89					1				1
88					1				1
87			1						1
83							1		1
78					1				1
77					1				1
76					1			1	2
74					1				1
72					1				1
67					1			1	2
66					2				2
65						1			1
	109	5	4	1	16	9	2	4	150

The table shows that although the bulk of the micrococci are definitely resistant to most of the Escherichia strains, nearly 10 per cent of them are relatively sensitive. Strain 60, which

also often grew better on staled agar than on the control plate, is the only one for which a clear increase in growth was noted in many cases by the antibiotic method. This should be considered as a probiotic phenomenon (Photo 2).

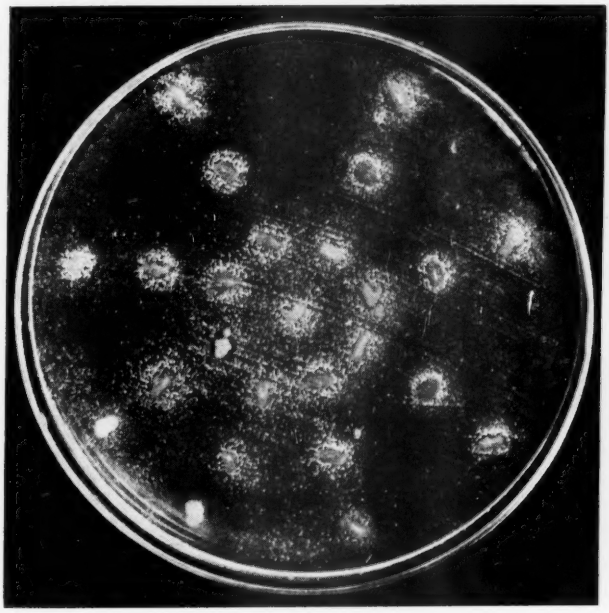


Photo 2. The probiotic effect of Escherichia on the growth of micrococcal strain 60. Micrococcal colonies are found almost exclusively in the surroundings of the Escherichia colonies.

Note, however, the Escherichia colony with an exclusively antibiotic effect at the bottom of the picture.

The table shows, further, that although the strains of Micrococcus pyogenes v.aureus generally seemed to be resistant and the strain of M.epidermis mainly sensitive, no correlation can be shown with any individual biochemical reaction, general sensitivity to antibiotics (186) or size of colony. Table 8 shows

that, whether the micrococci were isolated as mixed populations with Escherichia or alone, no statistical difference is noted in the resistance of these groups.

TABLE 8

Comparison of the resistances to the antibiotics produced by Escherichia of micrococci isolated from the same samples as Escherichia and from elsewhere

Value	Isolated together with Escherichia		Isolated separately from Escherichia	
	All	M.pyog.v.aur.	All	M.pyog.v.aur.
100	6	6	6	3
99	16	15	23	22
98	10	9	13	10
97	6	6	13	12
96	7	6	7	6
95	1	1	5	3
94	2	1	3	2
93	3	2	6	4
92	—	—	2	1
91	1	—	2	—
90	2	—	1	—
89	—	—	1	—
88	—	—	1	—
87	—	—	1	—
83	1	—	—	—
78	—	—	1	—
77	—	—	1	—
76	—	—	2	—
74	—	—	1	—
72	—	—	1	—
67	—	—	2	—
66	—	—	2	—
65	—	—	1	—
	55	46	95	63
	M = 97	M = 98	M = 94	M = 97

All ..... difference 3 ± 1.5
M.pyog.v.aur. ..... difference 1 ± 1.1

Escherichia Strains Producing Antibiotics Effective on Apathogenic Micrococci. — Table 9 shows that certain Escherichia strains have an antibiotic effect solely on apathogenic micrococcal strains. The inhibition zones formed by these strains were often much more extensive than those formed by the others whose inhibition zones usually did not exceed 3 mm. These Escherichia strains belonged to the typical E.coli strains and to one intermediate group (I — H_2S — A.c. +). The latter included all the representatives of this biochemical type in the present material and they proved to be the most potent producers of these inhibitors. The micrococcal strains sensitive to these inhibitors could be sensitive to other strains as well, whereas none of the micrococci considered as pathogenic were in the least sensitive to these strains of Escherichia.

TABLE 9

Escherichia strains effective on apathogenic micrococci only

Micrococci strains		Escherichia strains										
No.	species	VII	VIII	XIX	XXII	A_7	A_{44}	A_{45}	A_{46}	B_1	D_8	D_{15}
A_{22}	alb. apath.										x	
D_4	epid.	x	x	x	x	x	x	x	x	x	x	x
19	epid.	x	x	x	x	x	x	x	x	x		x
41	cand.	x	x			x	x		x			
48	epid.	x	x	x	x	w	w	w	w		w	w
51	epid.					x						
54	epid. g–	x	w	w	w	x	w	x	x	w	x	
57	epid.	x	x									
58	unid.	x	x	x	x	w	x	x	x			
59	unid.	x	w	x	x	w	w	x	x	x		
60	epid.			x+		w+	w	x				
61	epid.	x	w	x	x	w	x	x	x	w	x	x
66	epid.			x	x		x	x	x	x	x	
67	epid.	x	x	x	x	w	w	x	x	x		x
68	epid.	x	x	x	x	w	x	x	x	x	x	
70	epid.	x	w	x	x	w	w	w	w	x	x	x

x = inhibition zone 1 — 5 mm
w = inhibition zone 6 — 12 mm
+ = also increase in growth

COMPARISON OF RESULTS OBTAINED WITH THE STALED AGAR TECHNIQUE AND THE ANTIBIOTIC TECHNIQUE

— *Escherichia Strains.* — Fig. 2 shows the results obtained by the different methods giving the staled agar antagonism indices on the abscissa and the antibiotic indices on the ordinate. It will be seen that the results obtained by the two techniques are not comparable; however, if the strain had an antibiotic index exceeding the average, it was also strong in staled agar antagonism, and those that proved weak in the staled agar method were also weak in the antibiotic method. The bulk of the strains constitutes a uniform staled agar antagonistic group

Fig. 2. Diagram showing the relationship between the staled agar antagonism and the antibiotic antagonism of the Escherichia strains. The abscissa shows the staled agar indices, the ordinate the antibiotic indices. Value of each side of the square = 100.

with a low production of antibiotics. Some 10 per cent of the strains differ markedly from this average result.

A comparison of Escherichia strains isolated from diarrhoeal stools and from stools of healthy subjects shows, according to Table 10, that the 47 stools isolated from diarrhoeal stools were,

TABLE 10

Comparison of the antagonism of Escherichia strains isolated from diarrhoeal stools and from stools of healthy subjects on staled agar and using the antibiotic technique

Staled agar antagonism			Antibiotic production		
Index	Diarrhoeal stools	Others	Index	Diarrhoeal stools	Others
98	2	—	38	1	—
97	4	—	31	1	—
96	11	1	23	1	—
95	12	5	20	1	1
94	3	8	12	1	—
93	6	3	10	1	—
92	4	1	7	2	—
91	1	3	6	3	2
90	—	2	5	1	3
89	1	2	4	5	3
88	—	2	3	8	2
86	1	2	2	9	5
85	—	1	1	6	8
83	2	—	Under 1	6	4
67	—	1	0	1	3
	47	31		47	31
	M = 94	M = 91		M = 5	M = 3
	s = 3.3	s = 5.4		s = 7.7	s = 3.7
	difference 3 ± 0.97			difference 2 ± 2.2	

on the average, more strongly antagonistic in the staled agar technique. The difference 3 ± 0.97 between the average indices of the diarrhoeal strains, 94 ± 3.3, and of the non-diarrhoeal strains, 91 ± 5.4, by the staled agar technique, can be considered significant statistically.

The average indices obtained by the antibiotic technique were: diarrhoeal strains 5 ± 7.7 and non-diarrhoeal strains 3 ± 3.7; their difference, 2 ± 2.2, cannot be considered statistically

significant. The dispersion of the strain is found to be clearly greater in the antibiotic method than with the staled agar technique.

— *Micrococcal Strains.* — Fig. 3 shows the results obtained by the different techniques, the antibiotic resistance value being plotted on the abscissa and the staled agar antagonism resistance value on the ordinate. The diagram shows that the bulk of the strains constitutes a uniform group strongly resistant to antibiotics but sensitive to staled agar antagonism. However, the dispersion is greater than with Escherichia. For four strains,

Fig. 3. Diagram showing the relationship between the staled agar resistance and the antibiotic resistance of the micrococcal strains. On the abscissa are plotted the staled agar resistance values and on the ordinate the antibiotic resistance values. Value of each side of the square = 100.

the results obtained by the two techniques can be considered roughtly comparable, but with the remaining strains the results obtained with the different techniques are not compatible.

— *Comparison of Escherichia and Micrococcal Strains Isolated from One Specimen.* — Fifty pairs of Micrococcus and Eschericia strains in the material were isolated from one and the same specimen. The results obtained for these strains with the different techniques are shown in Table 11, which also gives the so-called

TABLE II

Comparison of Escherichia and Micrococcus strains isolated from one and the same specimen

No.	Micrococcus			Escherichia		
	a	b	c	a	b	c
A_1	43.3	90.5	66.9	83.1	1.5	*42.3*
A_2	4.0	98.5	51.3	95.1	1.0	48.2
A_4	3.0	97.8	50.4	95.1	6.2	55.7
A_5	2.0	99.0	50.5	96.6	3.7	50.2
A_6	21.7	90.0	55.8	95.8	0.7	48.3
A_7	4.0	100.0	52.0	92.9	2.0	47.5
A_8	1.3	100.0	50.7	95.8	7.2	51.5
A_9	14.3	100.0	57.2	97.1	3.7	50.4
A_{10}	1.0	97.8	49.4	93.8	4.2	49.0
A_{11}	1.0	96.3	48.7	96.6	20.5	**58.1**
A_{12}	1.0	96.5	48.8	96.2	23.3	**59.8**
A_{13}	8.0	97.5	52.8	91.1	0.5	*45.8*
A_{14}	0.7	100.0	50.4	89.6	4.3	46.9
A_{15}	24.0	96.8	60.4	86.9	6.0	46.9
A_{16}	0.3	97.5	48.9	83.3	3.2	*43.3*
A_{18}	2.3	93.0	47.7	96.2	2.8	49.5
A_{19}	2.0	96.8	49.4	93.8	38.3	**66.2**
A_{22}	36.0	83.3	59.7	95.1	0	47.6
A_{24}	2.7	99.5	51.1	95.3	2.3	48.8
A_{25}	2.7	100.0	51.4	98.0	1.3	49.7
A_{27}	3.0	100.0	51.1	98.0	2.5	50.3
A_{28}	1.0	99.8	50.4	93.6	3.5	48.6
A_{29}	0.7	99.8	50.3	96.4	2.2	49.3
A_{30}	16.7	98.0	57.4	97.3	0.5	48.9
A_{31}	1.7	98.8	50.3	94.0	2.3	48.2
A_{32}	1.7	98.0	49.9	96.2	1.5	48.8
A_{33}	1.7	95.8	48.8	94.9	1.8	48.5
A_{34}	9.7	94.8	52.3	94.0	10.3	**52.2**

TABLE II, cont'd

No.	Micrococcus			Escherichia		
	a	b	c	a	b	c
A_{36}	2.7	96.3	49.5	96.0	0.7	48.4
A_{37}	1.3	98.0	49.7	95.1	4.3	49.7
A_{38}	1.7	98.8	50.3	96.9	3.3	50.1
A_{39}	1.0	99.3	50.2	93.8	2.8	48.3
A_{40}	1.7	99.0	50.4	93.1	3.3	48.2
A_{41}	1.7	98.5	50.1	95.8	3.3	49.6
A_{43}	1.0	99.3	50.2	97.1	0.5	48.8
A_{45}	1.3	99.3	50.3	96.2	6.0	51.1
A_{46}	7.3	99.5	53.4	96.9	5.2	51.1
A_{47}	1.0	96.0	48.5	95.1	12.3	**53.7**
A_{48}	79.7	99.8	89.8	92.7	4.7	48.7
B_1	0.3	99.5	49.9	94.9	5.7	50.3
B_2	1.3	97.8	49.1	87.0	0	*43.8*
B_3	0.7	99.8	50.3	94.2	0.3	47.3
B_4	0.7	94.0	47.4	96.6	0.3	48.5
B_6	2.7	99.0	50.9	94.0	30.8	**62.4**
86	4.7	98.3	51.5	91.3	4.3	47.8
87	3.0	96.3	49.7	94.4	0.8	47.6
88	0.7	99.8	50.3	94.7	0.2	47.5
89	11.3	98.3	54.8	95.1	3.2	49.2
90	4.0	97.8	50.9	94.7	2.8	48.8
91	2.0	91.3	46.7	94.0	1.7	47.9

a = staled
b = antibiotic
c = total value = Ma.b.

86 esch. = XXIV
87 esch. = D_2
88 esch. = D_3
89 esch. = D_6
90 esch. = D_2
91 esch. = D_{10}

total antagonism or total resistance values, the arithmetic means of the values obtained by the two techniques. It is seen that when the Escherichia strains grown from the specimen show a high total inhibition index (A_{19}, B_6, A_{12}, A_{11}, A_{47} and A_{34}), the corresponding micrococci are not resistant but usually sensitive; and if the Escherichia strains are weak (A_1, A_{16}, B_2 and A_{13}) the micrococci may be either resistant (A_1), moderate (A_{13}) or sensitive (B_2 and A_{16}). The potency of the Escherichia strains isolated from identical specimens with

resistant micrococci (A_{48}, A_9, A_{30}, A_{22}) or with sensitive micrococci (91, B_4, A_{18}) varies independently of them.

Correlation of Micrococcal Resistance with Their Incidence on Isolation in Strains Isolated from Stools. — It could be assumed that at the primary isolation of the micrococci from the stools the resistant micrococci would be quantitatively more abundant than the sensitive micrococcal strains.

The quantitative incidence of micrococci in diarrhoeal stools was determined macroscopically on the basis of the number of colonies on a primary isolation plate containing potassium chromate. Table 12 shows the results obtained by the two

TABLE 12

Incidence of micrococci on isolation from stools compared with their resistance to Escherichia antagonism and antibiotics

Value	Staled agar resistance			Value	Antibiotic resistance		
	+	++	+++		+	++	+++
100	—	1	4	Under 1	1	—	—
99	2	4	4	1	6	4	8
98	3	—	2	2	2	2	1
97	—	—	2	3	—	—	2
96	2	2	1	4	—	—	2
95	—	—	1	5	—	—	—
94	1	—	—	7	—	1	—
93	2	—	—	9	1	—	—
90	1	—	—	14	—	—	1
83	1	—	—	36	1	—	—
				43	1	—	—
	12	7	14		12	7	14
	M = 95 (M = 98.) M = 98.				M = 8. (M = 2.) M = 3.		
	difference = 3 ± 1.2				difference = 5 ± 4.1		

+, ++ and +++ = incidence on isolation

techniques, classified by the quantitative incidence. The results obtained for resistance to staled agar antagonism justify the conclusion that no statistical correlation between resistance and quantitative incidence is elicited by either of the two methods.

DISCUSSION

From 1932 when F.E. Koch and coll. (141) found variations in the antagonism of the different Escherichia strains to micrococci, this antagonism was not studied in an extensive material until 1955 when Lavergne and coll. (149) published their report. However, their results were not known to the author when the present investigation was under way.

The staled agar method has been employed by many investigators of the antagonism phenomenon (e.g., 39, 83, 157, 168). Coblez and coll. (39) when using this method, took the sum of the values assigned for growth intensity of sensitive strains to indicate the strength of the antagonism. In the present work a similar determination was used to compute both the strength of the strains considered as antagonistic and the resistance of the sensitive strains. The stability control determinations effected justify the application of the value thus obtained.

McLeod and coll. (168) were the first to employ a technique similar to the staled agar method in studying the effect of E.coli on micrococci. They mixed E.coli culture with agar, poured plates and spread a thin sterile layer of agar on top. After the plates had set, the micrococci to be studied were inoculated into the surface. The E.coli had a partial inhibitory effect on the micrococci. Lockhard and coll. (157) claimed that M.pyogenes v.aureus did not grow at all on staled agar plates made of 48-hour E.coli culture. The present work demonstrates, however, that although the majority of the Escherichia strains completely inhibited most strains of M.pyogenes v. aureus, there are both weak Escherichia strains incapable of more than partial inhibition of M.pyogenes v.aureus, and resistant M.pyogenes v.aureus strains which grow moderately on most Escherichia strains. The results comply with those obtained by Koch and coll. (141) using Nissle's E.coli index technique, according to which the two genera show individual variations in relation to antagonism.

Like Fredericq and coll. (83), who pointed out that the staled agar method and the antibiotic method do not give identical results, the present material shows that the results generally differ. The effect of the different Escherichia strains on

micrococci show great variations. It seems obvious that, although the few biochemical determinations affected revealed no correlation with the intensity of the effect, the E. Freundii and intermediate strains are relatively stronger with the staled agar method and relatively less active with the antibiotic method than E.coli.

Zabalueva (237) claimed, on the basis of his index determinations, that the pathogenic aureus types are more resistant than the apathogenic, which again are more resistant than the albus types, while the citreus types are the weakest of all. The present material, however, does not support his claim if generalized, but shows that the sensitivity of micrococcal strains to the antagonism of Escherichia may be independent of their remaining qualities, except that the E. candidus strains studied seemed to be relatively sensitive with the staled agar method although not with the antibiotic method.

It might be assumed that micrococcal strains that have grown in vivo together with Escherichia strains would be more resistant to their antagonistic effect. However, no absolute difference is discernible among micrococci grown together with or separate from Escherichia in resistance to Escherichia in general or among micrococci grown together with Escherichia in resistance to strains isolated from the same specimen or to other strains.

Special attention is aroused by the occurrence in the material of Escherichia strains effective only on apathogenic micrococci. Considering the often particularly large inhibition zone found in them, they are obviously capable of producing a specific antibiotic active on apathogenic micrococci. Although these antibiotics in the present experiments did not demonstrably affect all the apathogenic micrococci, it might nevertheless be assumed that, if this active inhibitor could be isolated, it might be of importance for verifying the pathogenicity of and for identifying the micrococci. No absolute criterion is yet available for this purpose (185).

When the Escherichia strains isolated from diarrhoeal stools are compared with those isolated from the stools of healthy subjects, the slightly greater activity of the former attracts attention. As, according to Schiller (204), micrococci are seldom

found in the stools of healthy subjects but, according to Linz (155), are relatively often present in the stools of enteritic patients, the opposite assumption, viz. that diarrhoeal stools would reveal less markedly antagonistic Escherichia strains, suggests itself. According to Nissle (180), too, the E.coli strains should be weaker in the stools of pathologic cases.

If, in fact, the diarrhoeal strains are more strongly antagonistic to micrococci and if this is also true of their antagonism to other bacterial strains [it has been demonstrated that Escherichiae in general are antagonistic to other strains (141, 189, 201)] and provided the same factors are also effective in vivo in the intestine, this might perhaps throw light on the pathogenic properties of these Escherichia strains in the intestines. By destroying other bacteria of the normal flora and by gaining the position of predominant flora, they may be capable of producing disorders manifested as diarrhoea.

2
ANTAGONISM STUDIES ON SELECTED STRAINS

In order to investigate the part played by certain factors, assumed to produce antagonism, in the antagonism between the genera Escherichia and Micrococcus, Escherichia strains of varying antagonistic intensity and micrococcal strains of varying sensitivity were selected for further studies on the basis of the mass experiments reported in the early part of the present paper.

A. SOLID CULTURE MEDIUMS

COMPARISON OF MICROCOCCAL STALED AGAR AND ESCHERICHIA STALED AGAR

On the basis of the staled agar tests reported in Section I a number of sensitive (D_3, 19, 68, 88 and D_8), less sensitive (45, 2, 29, 76, A_{13} and A_{40}), and of resistant (7, A_2, A_{48}, 63 and 57) micrococcal strains, and Escherichia strains of weak antagonism (A_1, D_{17}, XV, XXXII, and A_{16}), of medium (XII, A_{31}, A_{34}, B_6 and D_{10}) and of strong antagonism (XI, A_{25}, A_{27}, A_{30} and A_{43}) were selected. The micrococci were

grown in the normal way for 24 hours in peptone water, and staled agar plates were made of them, on which the Escherichia were inoculated in the form of a zigzag line.

The antagonism of the Micrococci and the resistance of the Escherichia were determined from the size of the Escherichia strain colonies by the technique described earlier, though this time the following values were given for degrees of growth vigour: 0 = no growth; 1 = colony 0.5 mm or less; 2 = colony approx. 1 mm; 3 = colony approx. 1.5 mm; and 4 = colony approx. 2 mm or more. The results are given in Table 13.

TABLE 13

Effect of micrococcal staled agar on the growth of Escherichia. The activity index of the micrococcal strains compared with their resistance to the antagonism of the Escherichia and the resistance of Escherichia compared with the intensity of their antagonism on staled agar

Micrococci			Escherichia		
Strain No.	Antagonism of micrococcus	Resistance of micrococcus	Strain No.	Antagonism of Escherichia	Resistance of Escherichia
63	70.0	91.0	A_{27}	98.0	37.5
A_{48}	82.5	79.7	A_{25}	98.0	34.4
57	85.0 M = 77.2	51.3	XI	97.6	41.5 M = 38.2
A_1	79.2	43.3	A_{30}	97.3	35.2
7	69.2	34.0	A_{43}	97.1	42.4
2	67.5	9.3	A_{31}	94.0	53.2
29	71.5	9.0	A_{34}	94.0	38.3
45	62.5 M = 65.0	8.7	D_{10}	94.0	37.9 M = 38.5
A_{13}	65.0	8.0	B_6	94.0	34.4
76	58.3	8.0	XII	94.0	28.9
A_{40}	75.9	1.7	XXXII	85.8	43.1
88	65.8	0.7	A_{16}	83.3	39.1
D_3	46.7	0	A_1	83.1	37.5 M = 41.1
D_8	30.0 M = 44.4	0	D_{17}	67.8	49.8
68	28.3	0	XV	67.3	35.9
19	20.0	0			

The table reveals that with the staled agar technique the micrococci in many cases showed the greater antagonism the

more resistant they were to the antagonism of the Escherichia. However exceptions were found in strains 29, A_{40} and 88. By contrast, no definite correlation was noted between the resistance of the Escherichia and their antagonism. But, none of them grew normally on all the staled agar plates of micrococci.

SOME BIOCHEMICAL CORRELATIONS

Peroxide Production by Escherichia. — In order to find out the extent to which the observed antibiotic effect of the Escherichia was due to possible peroxide formation, attempts were made to determine the peroxide formation of Escherichia.

— (A) In a liquid medium: The Escherichia were grown for 1—3 days in a thin layer of culture medium in one set of the tests, and in culture medium aerated by compressed air pumped through sterile cottonwool in others (5). Possible peroxide production was ascertained by means of a fresh potato, and fresh acetic acid solution of benzidine (12). A pneumococcal culture grown in the same way was used as control. The pneumococcal culture showed a strong peroxide formation while none of the Escherichia revealed the slightest peroxide formation by this method.

— (B) On a solid medium: The Escherichia strains to be tested were inoculated into benzidine culture medium (188), as was the pneumococcal strain serving as control. All the pneumococcal colonies were completely black, while only strains XXIII, B_6 and B_2 showed a darkening around the colony, and strains XV and A_{19} a very slight darkish ring around the colonies; the rest of the Escherichia showed no darkening, although they all grew well. None of the Escherichia strains formed black colonies; hence they cannot be considered to form peroxide.

Alkali Formation by Micrococci on Peptone. — When grown on 10 per cent lactose agar slants (36), three strains were found to cause a slight reddening and strains 60, 63 and A_{48} a marked red colour, the alkali formed affecting the indicator contained in the medium. With the remaining strains, depending on

whether or not they fermented lactose and formed acids, the indicator colour turned yellow or remained unchanged.

Oxidase Content of Micrococci. — When fresh aqueous solutions of p-aminodimethyl-aniline monohydrochloride or tetramethyl--p-phenylendiamine hydrochloride were poured on agar cultures, strains 60, 63 and A_{48} (and, previously, strains 42 and 43) showed a marked positive oxidase reaction.

EXHAUSTION OF NUTRIENTS

If the loss of nutrients were of importance to the antagonism, a stronger inhibition should be obtainable by prolonged primary incubations of the peptone broth. To test this supposition, a series for staled agar antagonism was made with a weak (D_{17}), medium strong (B_6) and strong (A_{27}) Escherichia strain. The micrococcal strains were those used in the tests of micrococci on staled agar described above. The Escherichia were inoculated into five flasks containing peptone broth and incubated in the rotator. Plates were poured from the flasks after incubation periods of 6, 12, 24, 48 and 72 hours, and the micrococci were inoculated in the normal way after the plates had dried. The growth vigour of micrococci was, however, generally the same, independent of the primary incubation time, but in some cases may even have been more prominent on plates made from cultures incubated for a longer time.

In addition, as the medium contained in the 4 per cent agar employed in the above series may inhibit the effect of the nutrient loss, another test series was made in which the poured staled agar plates were incubated for 1/2, 2, 4 and 24 hours before the inoculation of micrococci. By this method the possible effect of nutrient loss should emerge clearly. The staled agar plates were made on Escherichia strains A_{30} and D_{17}. The results showed that with strain A_{30} the incubation of the plates did not affect the antagonism, but with the weakly antagonistic strain D_{17} the plate incubated for 24 hours showed a significantly better growth than the plates that had been incubated for a shorter period.

BACTERICIDIA — BACTERIOSTASIS

In order to find out whether the antagonism noted on staled agar was bacteriostatic or bactericidal, normal staled agar plates were made of the strongly antagonistic strains A_{27} and A_{30}, on which micrococci 1, 8, 19, 41, 45, 48, 61, 69, 78, 80, A_7, A_{16}, A_{18}, A_{45}, D_3 and D_{10} were inoculated. On plate A_{27}, the strains 1, 45, 48 and D_{10}, and on plate A_{30} the strains 1, 8 and 61 were found to have grown colonies barely visible to the eye during the 48-hour incubation period. The remaining strains had formed no colonies visible to the eye. A specimen was taken by a sterile loop from the initial end of the inoculation line, and inoculated into a blood plate containing potassium chromate. All the strains, apart from strains 19, 41 and 69 which did not grow at all, were found to grow normally in 24 hours.

ANTIBIOTICS

Antibiotics on Staled Agar. — In order to determine whether the antagonism noted on staled agar made of Escherichia culture depends on factors secreted into the culture medium or whether it is so-called direct antagonism bound with living cells, the following series of tests was carried out, the strains listed in Table 14 being selected as the experimental strains.

Three cultures were made of each Escherichia strain. After a normal primary incubation they were treated as follows:

(a) one part of the first culture was made in the usual way as a staled agar plate (whole), another part was centrifuged (by Sphinco ultra-centrifuge) for 30 min. at 20,000 RPM and a staled agar plate was made of peptone broth poor in cells (broth), and a third plate (cells) was made of sedimented cells, suspended in a volume of sterile saline equal to the centrifuged broth before they were mixed into plates.

(b) one part of the second culture was taken to make an ordinary staled agar plate (whole), the rest was centrifuged, and some of it was used to make a plate of peptone broth poor in cells (broth) as above, and a third plate (cells) was made on sedimented cells, which this time were suspended in fresh peptone broth.

TABLE 14

Strains of Escherichia and Micrococcus employed in the experiment

			staled		antib.	
			apathog.	pathog.	apathog.	pathog.
Escherichia	coli atyp.	I			weak	weak
,,	coli	XV	weak	weak		
,,	coli atyp.	XXIII		strong		
,,	intermedius	A_7			strong	
,,	coli	A_{19}				strong
,,	freundii	A_{20}	strong			
,,	intermedius	A_{30}		strong		
,,	intermedius	A_{43}	strong			
,,	intermedius	A_{44}			strong	
,,	intermedius	B_2			weak	weak
,,	coli	B_6				strong
,,	intermedius	D_{17}	weak	weak		
Micrococcus	p. v. aureus	3		resistant		
,,	p. v. aureus	7		resistant		
,,	epidermidis	19	sensitive			
,,	epid. gelat.–	54			sensitive	
,,	epidermidis	60	resistant			
,,	epidermidis	61			sensitive	
,,	unidentified	63			resistant	
,,	candidus	69			resistant	
,,	p. v. aureus	76				sensitive
,,	p. v. aureus	A_7				resistant
,,	p. v. aureus	A_{14}				resistant
,,	p. v. aureus	A_{16}		sensitive		
,,	p. v. aureus	A_{18}				sensitive
,,	unidentified	A_{48}	resistant			
,,	p. v. aureus	D_3		sensitive		
,,	epid. gelat.–	D_8	sensitive			

(c) the third culture was sterilized by ethylene oxide before further treatment. After 24 hours of primary incubation the culture flasks were placed for an hour in the refrigerator at $+4$ — $+$ 6° C. 1 per cent of liquid ethylene oxide was then added to kill the Escherichia, the mixture was shaken well and left for another hour in the refrigerator. After this the flasks were transferred to a thermostat at $+37°$ C where the ethylene oxide was allowed to evaporate overnight. Some of the culture

was then used to make an ordinary staled agar plate (whole), the rest was centrifuged as before, and a plate (broth) was made of peptone broth, and a third plate (cells) of sedimented cells suspended in fresh peptone broth. As a control to show that the ethylene oxide was in fact evaporated by the technique used and that any staled agar inhibition could not be due to it, uninoculated peptone broth was treated in the same way as the cultures, and added in the usual way into 4 per cent peptone broth to make plates.[1])

The results of the staled agars made with the different strains are given in Table 15.

TABLE 15

Antagonistic indices of staled agars made of living cultures, of the centrifuged medium of the cultures and of cells suspended in saline solution or peptone broth, and of those made of cultures killed by ethylene oxide, of medium centrifuged from them and of cells suspended in broth

Escherichia No.	whole			broth			cells		
	–	–	c	–	–	c	a	b	c
I	78.3	73.3	—	68.3	66.7	—	91.7	78.3	13.3
XV	66.7	60.0	—	66.7	48.3	—	88.3	58.3	10.0
XXIII	71.7	76.7	—	68.3	78.3	—	90.0	76.7	13.3
A_7	71.9	73.4	28.1	*42.2*	*45.3*	32.8	79.7	81.3	17.2
A_{19}	68.7	75.0	**57.8**	67.2	71.9	**85.9**	84.4	71.9	10.9
A_{20}	82.2	81.3	34.4	75.0	79.7	35.9	90.6	81.3	12.5
A_{30}	85.9	82.8	39.1	82.8	78.1	34.4	92.2	82.8	9.4
A_{43}	82.8	78.1	37.5	79.7	75.0	32.8	79.7	84.4	9.4
A_{44}	85.9	87.5	28.1	84.4	79.7	34.4	92.2	82.8	7.8
B_2	81.3	85.9	34.4	76.6	75.0	48.4	87.5	84.4	7.8
B_6	73.4	84.4	35.9	70.3	75.0	**53.1**	76.6	84.4	7.8
D_{17}	64.1	68.7	35.9	62.5	62.5	45.3	76.6	68.7	7.8
M =	76.1	77.3	36.8	70.3	69.6	45.4	85.8	77.9	10.6

whole = whole culture
broth = centrifuged culture medium
cells = centrifuged cells

a = suspended in saline
b = suspended in broth
c = cultures killed by ethylene oxide

[1]) A 1 cc specimen taken from the cultures treated with ethylene oxide in 10 cc peptone broth showed that the cultures had become completely sterile.

The table shows that on plates made of primary peptone broth (whole) the individual strains and the means of all the strains from living cultures gave identical results, whereas on the plate made of culture killed with ethylene oxide the antagonism was considerably weaker. An exception is made by strain A_{19}, which showed inhibition considerably exceeding the average even on the plate made of the killed culture.

The corresponding finding on plates (broth) made of centrifuged peptone broth was that the plates made of peptone broth centrifuged from living cultures gave identical results, and that the inhibition was greater than on plates made of peptone broth centrifuged from killed cultures. An exception is again made by strain A_{19} and, less markedly, by strain B_6; for A_{19} the inhibition on the plate made of the killed culture was even stronger than on those made of living cultures. It was also found that in all instances apart from strain A_7, the inhibition was nearly as strong on staled agars made of peptone broth alone as on plates made of living cultures of primary peptone broth.[1])

From plates (cells) made of centrifuged and re-suspended cells it was found that on suspension in saline solution (a), the inhibition obtained was often stronger than on suspension in peptone broth (b), which showed inhibition equal to that on plates made of primary peptone broth cultures. Hence the plates made of cells suspended in saline solution revealed the strongest antagonism of the whole series of experiments. An exception was made by strain B_6 with which the plate made of cells suspended in saline solution showed a clearly weaker inhibition than the plate made of cells suspended in fresh peptone broth.

Very little inhibition was noted on the plates made of killed cells; on the contrary, in most cases the micrococcal growth was markedly better than on the mere peptone agar plates serving as a control.

[1]) No Escherichia colony formation was observable inside the agar on staled plates made of centrifuged peptone broth; the plates remained clear throughout the incubation.

On plates made of sterile peptone broth after ethylene oxide treatment the micrococcal growth was quite normal, which indicated that the ethylene oxide left in the peptone broth after the treatment was not enough to inhibit the growth.

Colicines. — Had the antibiotic affect of Escherichia on the micrococci been due to colicines, the Escherichia with the strongest antibiotic effect on micrococci should also with the same technique have inhibited the growth of the Shigellae, as the latter as a rule are sensitive to the colicine effect. To test this, the following 16 Shigella type strains were challenged by all the Escherichia strains of the present material; the stab culture technique described in Section I was used.

8217 =	Shigella	dysenteriae	type 1 — Norwich (Sh. shigae)
8212 =	,,	,,	type 2 — 214 (Sh. Schmitzii)
8192 =	,,	flexneri	V — Kasauli
8204 =	,,	,,	WY — Schutz
8205 =	,,	,,	Z — Boyd 103
4130 =	,,	,,	Boyds P 119 — P 493
8207 =	,,	,,	(Newcastle) — Barry
8208 =	,,	boydii	170
8209 =	,,	,,	P 288
8210 =	,,	,,	(D. 1.) — 98 D. D.
8211 =	,,	,,	(D. 19.) — D. 27
8214 =	,,	,,	(P 247) — PSH 125
8212 =	,,	,,	(B 143) — 426 (FA)
6984 =	,,	,,	type 7
8220 =	,,	sonnei	phase I — Kasauli
8219 =	,,	,,	phase II — Wheeler

The results of the cases in which inhibition was observed are given in Table 16. It will be noted that only 16 Escherichia strains formed antibiotics which varyingly inhibited 10 different Shigella strains. Eighty four Escherichia strains were inactive, and five Shigella strains were resistant to all Escherichia strains.

Antibiotics Permeable through a Cellophane Membrane. — In addition, to find out whether the Escherichia possessed an inhibitory factor which could permeate a cellophane membrane,

TABLE 16

Sensitivity of Shigella strains to Escherichia strains, by antibiotic stab culture method

Shigella-strains No.	Escherichia strains															
	31	32	A_3	A_5	A_{14}	A_{15}	A_{19}	A_{21}	A_{24}	A_{38}	A_{40}	A_{45}	A_{48}	A_{49}	D_{10}	D_{18}
8204	—	—	—	—	—	—	3	—	—	—	—	—	—	2	2	—
8205	—	—	—	—	—	—	2	—	—	—	—	—	—	2	—	—
4130	1	1	—	—	—	—	4	4	—	—	—	—	—	3	—	1
8207	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—	—
8211	—	3	—	—	—	—	—	—	—	1	1	—	2	—	—	—
8214	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—
8212	3	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6984	4	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8220	4	—	3	—	2	2	—	5	—	1	1	—	—	1	—	—
8219	4	—	1	1	—	—	—	3	—	—	—	3	—	—	—	—

The figures indicate the inhibition zone in mm.

the following series of experiments was carried out. A cellophane membrane sterilized in water in an autoclave was spread on the surface of a plate 180 mm in diameter containing ordinary peptone agar. The plates were dried for half an hour at 37° C, after which Escherichia culture grown overnight was inoculated, by means of a sterile cotton pad dipped into it, into the middle of the plate over an area 30 mm in diameter. The plates were incubated for 24 hours at 37° C, after which the cellophane membrane was removed. The micrococci were inoculated in a zigzag line radially from the edge of the plate to the centre, 16 strains on each plate. The plates were again incubated for 24 hours at 37° C. The growth inhibition was graded as follows:

0 = no inhibition, normal growth also in the middle of the plate, or growth restricted for less than 2 cm from the centre of the plate,
1 = growth inhibited for 2-3 cm from the centre of the plate,
2 = „ „ „ 3-5 „ „ „ „ „ „ „
3 = „ „ „ 5-6 „ „ „ „ „ „ „, and
4 = „ „ „ over 6 cm from the centre of the plate or completely.

From the values obtained, the antibiotic strength of the Escherichia and the resistance of the micrococci and shigellae were computed by the method described before.

— Effect on Micrococci: The results are shown in Table 17, where they are compared with the antibiotic values obtained previously by the antibiotic technique, computed as if they were obtained from identical strains. However, it should be noted that the system of grading differed with the two methods; under the antibiotic method the figures indicated millimetres, here centimetres of inhibition.

The antibiotic effect of Escherichia in the cellophane membrane method was found in many cases to be relatively much stronger, and even appeared with strains (I, B_2) with which it was not observable by the antibiotic technique. However, the strains (B_6, A_{19} and A_7) most markedly antagonistic under the previous technique did not show the

TABLE 17

Effect of Escherichia on the micrococci by the cellophane membrane method, as compared with the "antibiotic technique"

Antibiotic strength of Escherichia

	Strain No.										
	A_{44}	XI	XXIII	D_{17}	B_6	A_{19}	A_{20}	A_{30}	A_7	XV	B_2	A_{43}
A	32.8	*29.7*	**26.6**	26.6	25.0	17.2	15.6	15.6	14.1	14.1	**14.1**	0
B	31.2	0	4.1	12.5	*47.9*	*45.8*	6.3	6.3	*29.1*	4.1	0	0

Resistance of micrococci

	Strain No.														
	63	A_{48}	A_{18}	A_{14}	A_{16}	7	D_3	A_7	3	76	69	60	D_8	19	61	54
A	100	100	97.9	95.8	95.8	93.7	91.8	**91.7**	89.5	87.5	**83.3**	79.2	58.3	47.9	39.5	37.5
B	100	97.7	95.8	100	93.7	97.1	89.6	100	89.6	87.5	100	79.1	89.6	70.8	60.4	64.5

A = cellophane membrane
B = antibiotic technique

TABLE 18

Effect of Escherichia on the shigellae by the cellophane membrane method as compared with the corresponding results with micrococci

Antibiotic effect of Escherichia

	Strain No.											
	A_{44}	I	XXIII	D_{17}	B_6	A_{19}	A_{20}	A_{30}	A_7	XV	B_2	A_{43}
Shigellae....	21.7	14.1	*90.6*	25.0	**68.8**	**68.8**	21.9	12.5	*78.1*	21.9	18.8	**42.2**
Micrococci ..	32.8	29.7	26.6	26.6	25.0	17.2	15.6	15.6	14.1	14.1	14.1	0

strongest inhibitions by this technique. On the other hand, considered from the point of view of the micrococci, the results show a clear correlation with the results obtained by the previous technique, with the exception of strains A_7 and 69, which were definitely more sensitive with this technique.

— Effect on Shigellae: The antibiotic effect of Escherichia on the shigellae, when the cellophane membrane method was used, compared with the corresponding effect on the micrococci, is shown in Table 18.

The table shows that the maximum inhibition on the shigellae was by no means produced by the same strains that produced the maximum effect on the micrococci. Escherichia strains XXIII and A_7, with a marked inhibitory effect on shigellae, reveal no particular inhibition on micrococci. Similarly, strain A_{43}, which was previously found to have no antibiotic effect at all on micrococci, gave a comparatively marked inhibition on shigellae. Only strains B_6 and A_{19}, which with the antibiotic technique proved to be most strongly antibiotic to the micrococci, were clearly antibiotic to the shigellae as well.

DISCUSSION

McLeod and coll. (168) and Lochard and coll. (157) had reported previously that micrococci are unable to inhibit the growth of Escherichia when the staled agar technique is used. From the present investigations, however, it is obvious that the micrococci, especially those capable of growing well on Escherichia staled agar, often inhibit the growth of Escherichia, even completely. The weakest micrococci, on the other hand, hardly inhibit the Escherichia at all. From this it could be assumed that the resistance of strong micrococci on staled agar might be due to their inhibiting effect on Escherichia. Both M.pyogenes v.aureus (87) and M.epidermis (160) have been found to be capable of producing antibiotics with some inhibitory effect on Escherichia. Had the inhibitory and resistant strains in the material had an antibiotic effect on staled agar, they would presumably have inhibited the growth of Escherichia colonies under the antibiotic technique. However, in the whole material of 100 micrococci, this was found only in a couple of

exceptional cases. On the other hand, the resistance of micrococci on Escherichia staled agar might also be attributed to a possible production by micrococci of proteases or a similar factor destroying any inhibitors produced by Escherichia. In that case, however, the inhibitory effect of the resistant micrococci on micrococcal staled agar could not be attributed to the same factor. Hence it is possible that both these assumed factors contribute to the micrococcal resistance and the inhibitory effect.

The determinations of peroxide formation effected on Escherichia indicate that with some strains at least, such as A_{44} which produces antibiotics effective primarily on apathogenic micrococci, true inhibitors classifiable as antibiotics are involved and not peroxide which has often been reported as the causative agent of the claimed antibiosis (24). Nor is any markedly strong peroxide formation, like that of the pneumococcus strain, found with the other strains classified by the antibiotic technique among the producers of antibiotics, e.g. A_{19} and B_6. It was impossible to show whether or not the darkening of the culture medium in the surroundings of these colonies was perhaps due to a slight peroxide production (nothing similar was found with the pneumococci, and the colonies themselves were completely black). Although the micrococci are strong producers of catalase (167), they have been found particularly sensitive to the effect of peroxide (12). Hence this question remains open.

The alkali formation of resistant micrococcal strains observed coincidentally in 10 per cent lactoce agar slants showed that they evidently are among the strongest ammonia-producing strains of their genus. Berdnikow (11) found that the strains most capable of decomposing proteins are more active antagonistically than the strains less capable of it; consequently, these strains showed strong inhibition of Escherichia on staled agar. If, again, the inhibitory effect of Escherichia on staled agar is assumed to be due to the formation of inhibitors similar to the antibiotics, as experiments have shown, the resistance of strong micrococcal strains could perhaps be attributed to their proteolytic ability, in other words, to their destructive effect on the inhibitor. The present work did not reveal whether

or not the oxidase-positive reaction discovered indicates that a possible destruction of inhibitors by oxidation constitutes an additional factor present in the most resistant strains.

If the antagonism of Escherichia on staled agar were due to loss of nutrients alone, the micrococcal growth could be assumed to be the weaker the shorter the primary incubation, or if the staled plates were incubated before the inoculation of the micrococci. However, the experiments made indicate that the antagonism decreases rather than increases in both tests as the period of incubation is increased. Hence antagonism is hardly based on exhaustion of nutrients. Wynne and coll. (236) showed that the antagonism of the genus Klebsiella was a result of a labile, specific inhibitory metabolite. Its production can be assumed to have reached its maximum after the termination of the logarithmic phase of cell multiplication, which with Escherichia occurs after approximately 12—24 hours, and to diminish later on. Hence the experiments made do not exclude the existence of such a labile inhibitor in the antagonism of Escherichia to micrococci.

As the subcultures taken from staled plates show, the inhibitory effect of Escherichia is primarily bacteriostatic, although on a particularly sensitive micrococcal strain it may even be bactericidal. This also speaks against nutrient loss being characteristic of antagonism and is reminiscent in the first place of the effect of toxic substances, where bacteriostasis goes over into bactericidia with increasing concentration or with more sensitive strains.

In the series of experiments using normally grown peptone broth cultures, peptone broth centrifuged from them, and centrifuged and re-suspended cells for the preparation of staled agar, it was found that centrifuged peptone broth gave a clear and nearly as strong inhibition as the plates made of bacteria-containing cultures. It may even be said that the stronger the inhibition with cultures containing bacteria, the stronger the inhibition with the peptone broth centrifuged from it. Hence the antagonism observed on staled agar must be due not only to inhibitors demonstrable by the antibiotic technique, but also to other inhibiting factors demonstrable in the mediums. This may also account for the fact that the staled agar technique

gives results not identical with those of the antibiotic technique, as was pointed out previously.

However, strain A_7 showed exceptionally little inhibition on plates made of centrifuged peptone broth. Were Escherichia to include strains with an effect similar to direct antagonism, this strain, on staled agar, would be primarily of that type in its inhibition to M.pyog.v.aureus. A_7, which was found to produce antibiotics effective on apathogenic strains, showed strong inhibition of these strains also on plates made of centrifuged peptone broth.

As the effect of suspended living cells was in many cases stronger than that of a whole culture, the factors inhibiting micrococci can be assumed to be produced primarily when bacteria multiply rapidly. Plates made of living normal cultures always contain autotoxic factors, whereas on plates made of centrifuged, re-suspended cells these factors are non-existent, when the Escherichia resume growth on the staled agar, and accordingly the growth is free from detrimental factors.

The stronger inhibition on plates made of cells suspended in saline solution as compared with those made of cells suspended in fresh peptone broth can also be attributed to loss of nutrients. On the latter plates the amount of nutrients will be twice that in the former. An exception, however, is made by strains B_6, with which the inhibition on the plate made of cells suspended in saline solution was weaker than on the plate made of cells suspended in fresh peptone broth. Perhaps the antibiotic produced by it is particularly dependent on the amount of nutrients available in the medium.

Carlson and coll. (31) are among those who state that ethylene oxide is suitable for sterilizing bacteria-containing solution. The 1 per cent ethylene oxide concentration employed could be shown to have a completely sterilizing effect on cultures that had grown for 24 hours. As regards the inhibitions observed on plates made of cultures treated with ethylene oxide, it may be said that ethylene oxide, a strongly active substance, evidently destroys inhibitors to some extent, as inhibition was stronger with the peptone broth centrifuged from living cultures. Strain A_{19} was exceptional, as ethylene oxide evidently did not affect its inhibiting factors.

The present series of experiments justifies the conclusion that the Escherichia in any case produce inhibiting factors soluble in the culture medium, that the loss of nutrients remains a secondary factor, and that the part played by direct antagonism is hypothetical.

Several authors have used a sterile cellophane membrane placed on the agar surface to demonstrate antibiotic formation (106, 124). This method can only prove the existence of inhibitors that penetrate the membrane. When left to grow for 24 hours undisturbed by other factors, the cultures often showed a stronger formation of inhibitors than with the stab culture antibiotic technique employed previously, under which the factors affecting the growth of the micrococcal strain and those produced by the micrococci and possibly destroying the inhibitors of Escherichia act simultaneously. Hence, as regards the antibiotic effect of Escherichia, the divergence between these results and those obtained by the antibiotic technique is not surprising. It may be pointed out that no major differences are observable between the antibiotic and cellophane membrane techniques as regards the sensitivity of micrococci.

Colicines have also been claimed to be effective on micrococci in some cases (42). According to the literature the shigellae are especially sensitive to colicines (79). Should the Escherichia strains in a material selected at random, with some effect on all micrococci or with an effect on apathogenic micrococci, produce colicines, and should their effect on the micrococci be based on the colicine effect, it may be supposed that these same strains would also inhibit the growth of shigellae. But from the experiments with shigellae we found that only strains A_{19} and A_{49} (Escherichia strains isolated from one and the same patient at different occasions) inhibited both the micrococci and some shigellae; these Escherichia strains had an effect on most of the micrococci. None of the Escherichia strains effective on the apathogenic micrococci inhibited the growth of the shigellae. The low number of strains inhibiting the shigellae in the material, however, gives rise to a suspicion that the technique employed was not the best possible for verifying the colicine effect, although the medium obviously was suitable for their formation (21). If the colicine is taken to affect enterobacteria alone (75), the

Escherichiae probably produce other inhibitors as well, which are effective both on micrococci and shigellae, and this may account for the contradictory reports in the literature on the antibiotic spectra of the colicines (20, 42, 75). In any cases it seems obvious that the antibiotic effect of Escherichia on the micrococci is not based on colicine effect.

B. LIQUID CULTURE MEDIUMS

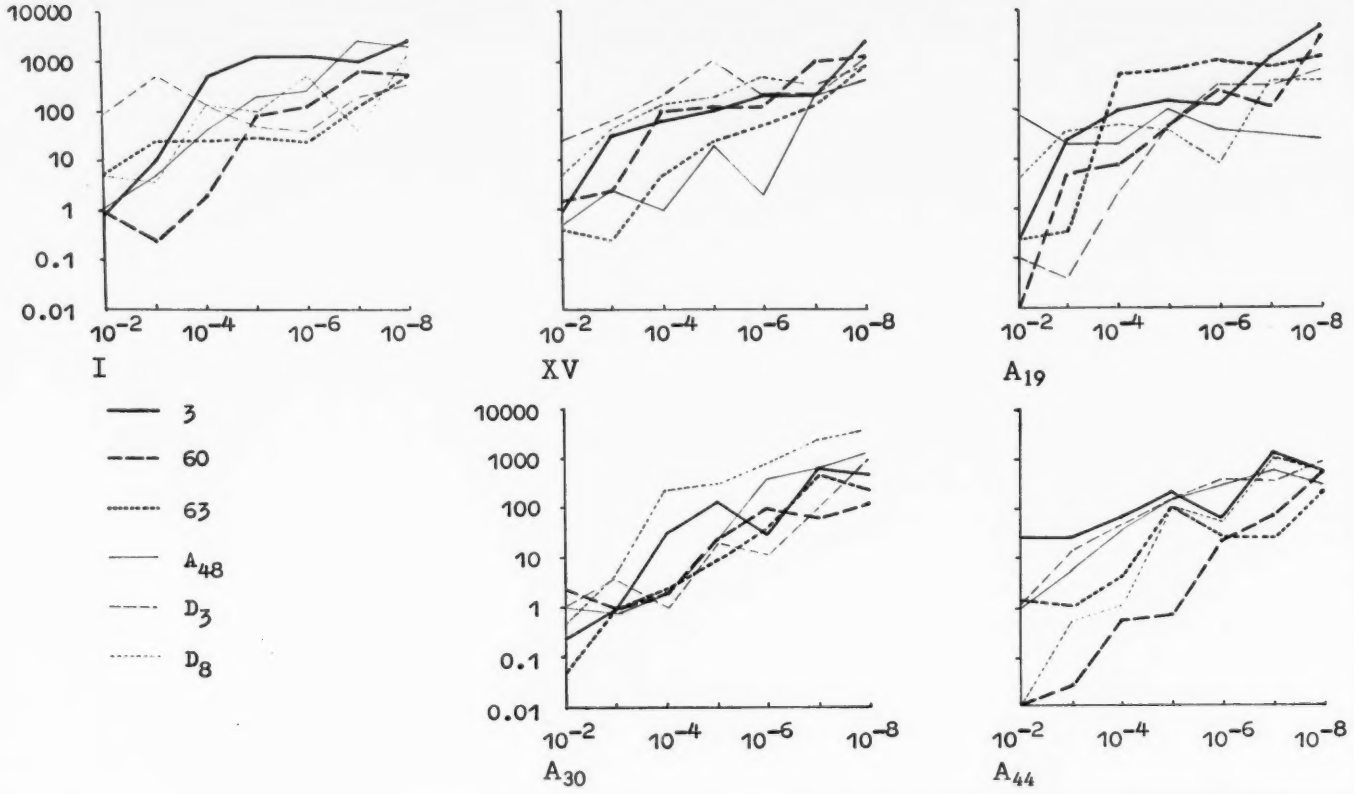
Further experiments were carried out in a liquid culture medium with the selected strains of Escherichia and Micrococcus of Section A, in order to arrive at quantitative bacterial determinations.

EFFECT OF CONCENTRATION ON INOCULATION

In order to find out whether the amounts of bacteria inoculated played a part in the manifestation of antagonism, the following series of experiments was carried out. Bacterial inoculation, in serial tenfold dilutions of Escherichia strains that had grown overnight and been diluted 1:100 (saline), was made into tubes containing 9 ml of peptone broth. 1 ml of micrococcal growth that had grown overnight and been diluted 1:1000 (peptone broth) was added to each tube, and the tubes were incubated overnight at +37° C. The next day the tubes were shaken well and diluted 1:1,000, 1:100,000, and 1:10 million. Using a glass elbow rod, 0.03 ml of each dilution was spread on the surface of a selective blood plate containing potassium-chromate, to enable the living micrococci to be counted. A control for the verification of the Escherichia was taken simultaneously by a loop from a couple of the last diluted tubes and transferred to ordinary peptone agar.

Prolific growth of Escherichia was noted on all the control plates with Escherichia. The number of living micrococci per 1 ml of undiluted culture was estimated by counting the colonies on the plates with the lowest dilution permitting an accurate count. The results are given in Figs. 4 and 5.

It was found that the larger the primary inoculation of Escherichia in proportion to the micrococci, the smaller as a rule was the number of micrococci surviving after 24 hours.

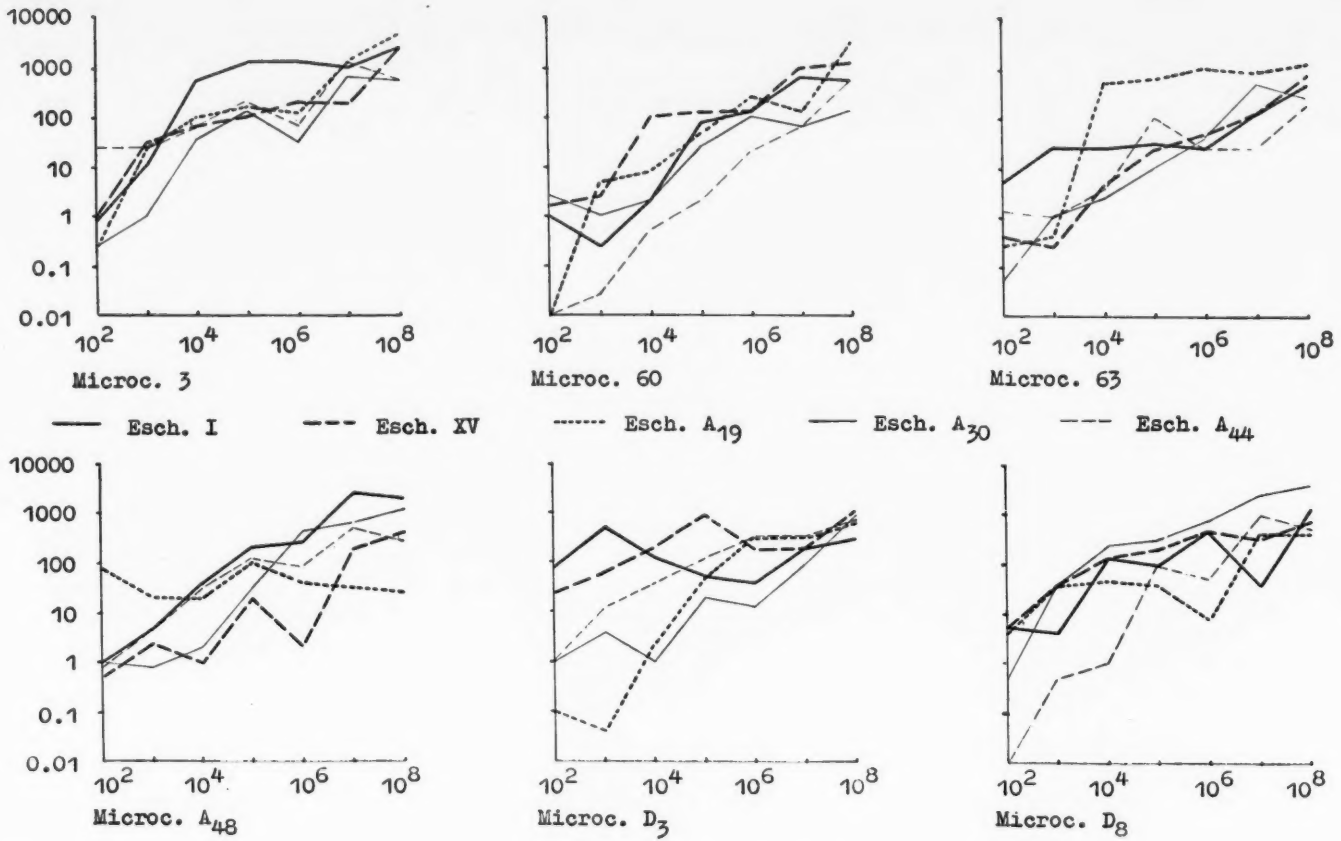

10000
1000
100
10
1
0.1
0.01
10^{-2}
10^{-4}
10^{-6}
10^{-8}
I
XV
A_{19}
3
60
63
A_{48}
D_3
D_8
A_{30}
A_{44}

In no case, however, had the micrococci disappeared within the incubation period of 24 hours, nor was complete disappearance noted, even when the cultures were observed for a longer period, after 72 hours of incubation.

Considering the results from the point of view of the various Escherichia strains (Fig. 4) it was found that for strains A_{19} and A_{44}, which are among those producing demonstrable antibiotics on agar, large Escherichia concentrations showed the maximum differences in inhibition. Escherichia strains I and XV, previously verified as weakly antagonistic, did not, even with high Escherichia concentrations, achieve as strong a reduction in the number of living micrococci as the rest of the strains tested. Strain A_{30}, which had not produced demonstrable antibiotics on agar but was strongly antagonistic on staled agar, reduced the amount of living micrococci by roughly equal proportions in all the micrococcal strains.

A review of the results from the point of view of the various micrococcal strains (Fig. 5) showed that strain 3 of M.pyog. v.aureus, most resistant on staled agar, and the apathogenic strain A_{48} revealed the least reduction in living bacteria. The weakest effect on the former was exerted by Escherichia strain A_{44}, which produced antibiotics effective on apathogenic micrococci. Micrococci 60 and 63, strains which were also resistant on staled agar, were definitely more sensitive than the former two by this technique, especially strain 60, which was particularly sensitive to the antibiotic-producing Escherichia strains A_{19} and A_{44}. Strain D_3, one of the sensitive M.pyog. v.aureus strains, was in this instance as well clearly more sensitive to strain A_{19} which produces antibiotics effective on the aureus strains, but it was also relatively sensitive to strain A_{44}, which produces antibiotics effective on apathogenic micrococci. With this technique, apathogenic micrococcus D_8

Fig. 4. Diagram showing the influence of the primary incubation of the different Escherichia strains on the growth of six micrococcal strains in a mixed population during 24 hours. On the abscissa are plotted figures indicating the dilution of the Escherichia culture at the moment of incubation, on the ordinate the amounts of living micrococci verified after 24 hours of incubation.


10000
1000
100
10
1
0.1
0.01
10^2
10^4
10^6
10^8
Microc. 3
Microc. 60
Microc. 63
Esch. I
Esch. XV
Esch. A_{19}
Esch. A_{30}
Esch. A_{44}
Microc. A_{48}
Microc. D_3
Microc. D_8

was one of the most sensitive, and in particular Escherichia strain A_{44}, which produces antibiotics effective on apathogenic micrococci, was inhibiting it in high concentrations.

DIFFERENT STAGES OF INCUBATION

The following series of experiments was carried out to ascertain whether mixed cultures showed antagonism at the initial stages of incubation. A culture of Escherichia and Micrococcus, grown overnight and suitably diluted, was inoculated into two flasks; an attempt was made to obtain a primary inoculation of approx. 100 bact./ml. One of the flasks was incubated in the rotator while the other stood at rest on the shelf of the incubator. Specimens were drawn from the flasks after 1, 2, 3, 4, 5 and 24 hours of incubation. 0.03 ml of each sample, or if preliminary tests showed it to contain a too high concentration of bacteria, 0.03 ml of the approximately diluted sample[1], was spread on a blood plate containing potassium chromate, to make possible a count of the living micrococci. To verify the number of Escherichia, 1 cc was drawn after 5 and 24 hours of incubation, suitably diluted with sterile saline solution, and filtered onto a grid millipore filter 4.5 cm in diameter, which was transferred onto an adsorbent pad, impregnated with sterile methylene green culture medium (70) and placed in a sterile Petri dish. This was then incubated at + 37° C in a humid atmosphere. After 24 hours of incubation the micrococcal colonies were counted from the chromate plate and the Escherichia colonies from the filter. As no essential change in the increase of micrococci was noted during incubation periods of 1—5 hours, Figs. 6 and 7 only give the results obtained from 1-hour, 5-hour and 24-hour cultures. Each strain was grown individually at the same time and in the same way; chromate

[1]) The dilution was made with peptone broth containing 0.5 per cent of potassium chromate.

Fig. 5. Diagram showing the development of the different micrococcal strains in a mixed population with five Escherichia during a 24-hour incubation period.

For values, see Fig. 4.

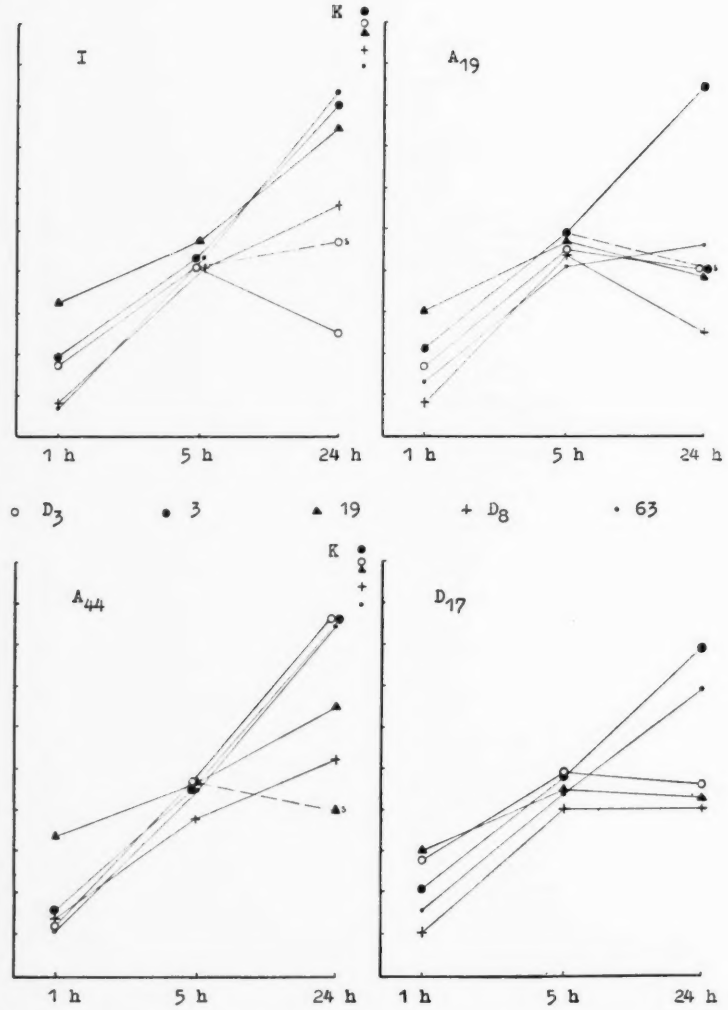


Fig. 6. Diagram showing the growth of micrococci in a mixed population with Escherichia at the various stages of incubation, seen from the point of view of the Escherichia. On the abscissa, the incubation period; on the ordinate, the number of living micrococci verified. K = the number of living cocci verified from micrococcal pure cultures, s = the result when grown in a rotator (in the event it differed clearly from those grown at rest).

plates were made of the micrococcal cultures and methylene green filter cultures from the Escherichia cultures, as above. The normal growth of the micrococci in a 24-hour culture is shown in Figs. 6 and 7 together with the results from mixed cultures.

When the determinations were repeated several times, the results obtained varied in details, mainly due to the diluting and to technical errors arising from the grouping of the micrococci — errors that cannot be eliminated. The results illustrated in the figures were those obtained from a series of experiments. The largest deviations from the normal were repeated in all determinations and this established a general trend.

The Eshcerichia numbers revealed no major changes compared with the pure culture controls; however, cultures incubated for 24 hours in the rotator generally showed higher bacterial amounts than the cultures at rest; this applies both to the mixed cultures and to the pure cultures. With the micrococci the opposite was the case, as with the mixed cultures in general fewer micrococci were found in the cultures incubated at rest than in those grown in the rotator, whereas in pure cultures the number of micrococci too was higher in cultures grown in the rotator than in those grown at rest.

A review of the results from the point of view of the various Escherichia strains shows that Escherichia strain A_{19}, a potent producer of antibiotics demonstrable on agar, had after 5 hours of incubation in mixed cultures definitely either destroyed living micrococci or considerably slowed down their reproduction. On the other hand, Escherichia strain A_{44}, which produces antibiotics effective on apathogenic micrococci and demonstrable on agar, had actually not inhibited the reproduction of strains 3 and D_3 of M.pyog. v.aureus or of the apathogenic resistant strain 63, whereas it had considerably inhibited that of the apathogenic sensitive strains 19 and D_8. Escherichia strains I and D_{17}, which proved weakly antagonistic both with the staled agar and the antibiotic technique, in these culture experiments inhibited the reproduction of the apathogenic strains 19 and D_8 and of M.pyog.v.aureus strain D_3 to a surprising extent.

A review of the results from the point of view of the micrococci showed that the number of living bacteria in the 5-hour

o I
● A_{19}
▲ A_{44}
+ D_{17}
D_8
K ·
1 h
5 h
24 h
63
K ·
1 h
5 h
24 h

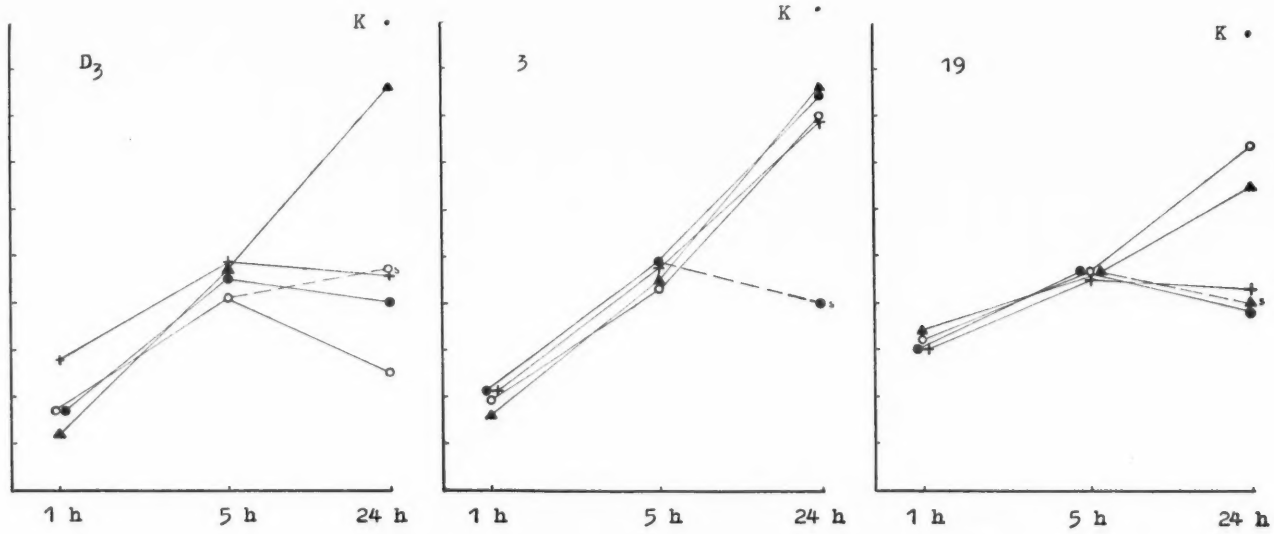


Fig. 7. Diagram showing the micrococcal growth in a mixed population with Escherichia at the different stages of incubation, seen from the point of view of the micrococci.
For explanation, see Fig. 6.

culture was as a rule roughly the same as in the pure culture controls; a considerable difference from the pure culture controls was noted only in the 24-hour cultures. In many cases the number of micrococcal colonies found in the 24-hour cultures was even less than in the 5-hour cultures, which indicates a direct decrease in the number of living micrococci. M.pyog. v.aureus strain 3, however, proved more or less resistant to all Escherichia apart from Escherichia A_{19} grown at rest. Micrococcal strain 63, previously proved to be a resistant apathogenic strain, was fairly resistant to all the strains. M.pyog.v.aureus strain D_3, sensitive on staled agar, proved sensitive in this experiment as well to all except A_{44}, an Escherichia strain which produces antibiotics demonstrable on agar and effective on apathogenic micrococci. The apathogenic micrococcal strains 19 and D_8, previously found to be sensitive, were generally sensitive in this instance, too, the amount of living micrococci found in 24-hour cultures being usually less than that in samples taken from 5-hour cultures.

EFFECT OF FILTRATES

In the previous chapter (A) it was reported that peptone broth separated from centrifuged culture had an inhibitory effect on micrococcal growth on staled agar plates. By contrast, no inhibition was obtained on micrococcal cultures in peptone broth in tubes with cultures filtered from Escherichia cultures after 24—120 hours of incubation, irrespective of whether the filtrates were made by Seitz or millipore filters.

However, active filtrates were successfully produced by the following technique (166). The Escherichia were grown overnight in a liquid culture medium, and 4 cc of the culture were added to a culture medium of 1.25 per cent of agar (Merck) in peptone broth in Roux's bottle. The bottles were incubated for 24 hours at 37° C, after which they were transferred into a deep-freeze box at —20° C for 2—7 days. After this they were transferred to a refrigerator at +4 — +8° C to defrost; the liquid medium was separated from the agar in 1—2 days. The liquid part was

then separated, immediately centrifuged for half an hour at 15,000 RPM in a cooled centrifuge, and the supernatant was collected. If the supernatant could not be dealt with immediately it was deep-frozen, and again unfrozen in the refrigerator when required. The unfrozen medium in which living Escherichia were still found was filtered through a millipore filter and titrated immediately in serial twofold dilutions by batches of 0.5 ml into test tubes. 0.5 ml of micrococcal culture grown overnight and diluted with saline solution 1:10,000 was added to the tubes, which were incubated for 24 hours at +37° C. The controls consisted of a tube containing the filtrate alone and another containing an amount of peptone broth equal to that of the micrococcal dilution alone. When the tests were repeated with new filtrates, the results were not identical.

The experimental series showed that Escherichia strains XV and D_{17}, in filtrates made as described, did not even partially inhibit the growth of any one micrococcus; strains A_7, I, A_{20}, B_2 and B_6 inhibited some of the micrococci, the titres being $^1/_2$—$^1/_4$; strains A_{19}, A_{30} and A_{43} inhibited several strains, the titres varying from $^1/_4$—$^1/_{16}$; XXIII inhibited most of the strains with these same titre values, and A_{44} inhibited only the growth of apathogenic micrococci, the titres varying from $^1/_2$—$^1/_8$. The most sensitive among the micrococci were strains A_{16}, D_3 and D_8, inhibited by titre values from $^1/_4$—$^1/_{16}$; strains 19, 54 and 61 were also sensitive to several filtrates, with titre values of $^1/_2$—$^1/_4$, whereas strains 3, 7, 63, 76, A_7, A_{14}, and A_{18} were only inhibited by some of the filtrates of the most potent Escherichia strains in $^1/_2$ dilution.

In order to find out whether mixed culture filtrates showed any stronger inhibition, indicative of adaptation antagonism, than the pure culture filtrates, the following series of experiments were made.

Seitz or millipore filtrates were made from mixed cultures and shown to have no inhibitory effect on the micrococci.

By the technique described above — — culture in Roux's bottles with deep-freezing, unfreezing, centrifuging and filtering — — filtrates were made from the following cultures:

Escherichia strains I and A_{19} in pure cultures,
Micrococci 3 and D_3 in pure cultures,
Escherichia I and Micrococcus 3 in mixed culture,
Escherichia I and Micrococcus D_3 — —,,— —
Escherichia A_{19} and Micrococcus 3 — —,,— — and finally
Escherichia A_{19} and Micrococcus D_3 — —,,— —

Thus, two Escherichia strains and two micrococcal strains were tested by the filtrates obtained. The results are given in Table 19.

TABLE 19

Effect of filtrates from pure and mixed cultures grown in semisolid culture medium on the growth of micrococci and Escherichia

Filtrate made of culture	Experimental strains			
	3	D_3	D_{19}	I
3	1/2 (1/4)	1/2	1/2	1/2
3 + A_{19}	1/2	1/2	1/2 (1/4)	1/4
3 + I	1/2	1/2 (1/4)	1/4	1/64
D_3	1/2 (1/4)	1/4	1/4	1/4
D_3 + A_{19}	1/2	1/2	1/4	1/2 (1/4)
D_3 + I	1/2 (1/4)	1/2 (1/4)	1/2 (1/4)	1/4
I	1/2	1/2 (1/4)	1/2	1/32
A_{19}	1/2	1/2 (1/4)	1/2 (1/4)	1/2 (1/4)

The figure indicates the inhibitory titre of the filtrate.
Partial inhibition given in brackets.

The table shows no greater activity in mixed culture filtrates than in pure culture filtrates for the micrococci. The effect of Escherichia filtrates on micrococci is of the same order as that of micrococcal filtrates. By contrast, Escherichia strain I was strongly inhibited by the mixed culture filtrate I+3, as also by the homologous filtrate. Hence nothing suggestive of adaptation antagonism was noted.

PARABIOTIC ANTAGONISM

In order to investigate parabiotic antagonism, the strains studied were grown on the two sides of a semipermeable membrane. In the first series of experiments the menbrane was in a

flask containing peptone broth; the membrane bag also contained the same medium. The Escherichia strains were inoculated outside the bag and the micrococci inside it. After 24 hours of incubation the micrococcal growth was found to be completely normal.

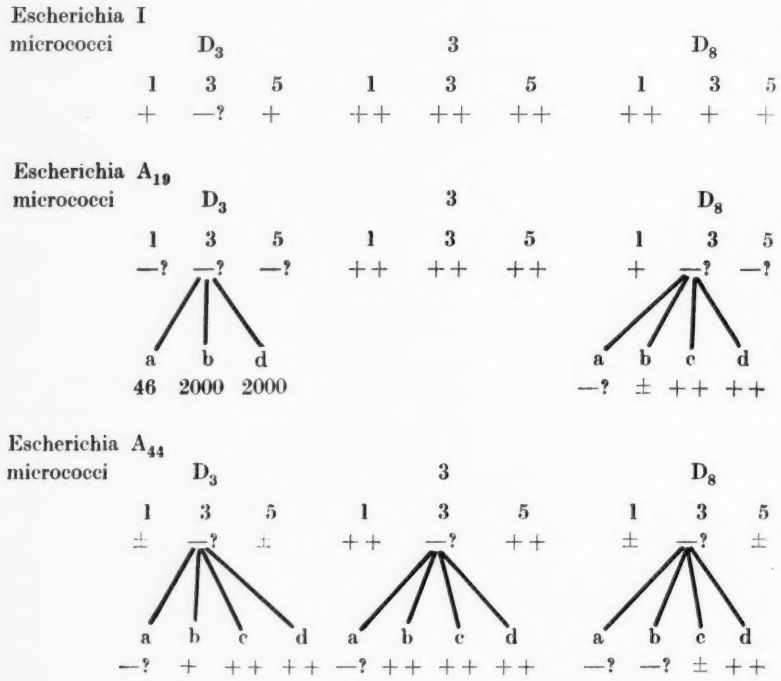
In the second series of experiments the Escherichia were allowed to grow in the outer culture medium for 1—7 days before the micrococci were inoculated into the bag. Again it was found that the reproduction of the micrococci in the bag was fully comparable to the normal.

In the third series of experiments the membrane bag filled with peptone broth was suspended in an empty flask and the Escherichia strains were inoculated on the external surface of the membrane. The inoculation of micrococci inside the bag was effected after 1, 3 and 5 days of incubation. After 24 hours the growth of the micrococci was checked by an anoptral (Reichert—Wilska) microscope, and if few cocci were seen the contents of the bag were stirred and a fresh sample drawn. When no growth was observed, some of the bag's contents were transferred into fresh bags, the first of which was empty inside and outside, the second with sterile saline solution on the outside, and the third fresh broth on the outside. Some of the culture was left in the original bag. After another 24 hours of incubation samples were drawn from the bags and the relative incidence of the micrococci was verified by an anoptral microscope and in one case by a surface count on chromate-containing plates. The results are given in Table 20.

The results reveal that Escherichia strain I inhibited the growth of Micrococcus D_3 in one case only. Escherichia A_{19} inhibited the growth of sensitive micrococci only, and on transfer into a bag surrounded by saline solution or fresh culture medium the growth returned to normal, although the original bag showed no growth during the second day. Escherichia A_{44}, although in the earlier experiments it had proved effective only on apathogenic micrococci, inhibited the growth of all three micrococci, but the growth returned to normal or nearly normal in the bags surrounded by air, by saline solution or by culture medium, while in the original bag the inhibition remained unchanged.

TABLE 20

Growth of micrococci in a bag of semi-permeable membrane outside which Escherichia is grown



a = original bag
b = bag surrounded by air
c = saline solution on the outside
d = culture medium on the outside
The figures indicate the number of colonies on the chromate plate.

—? = a couple of cocci in the field of vision
± = a few groups in the field of vision
\+ = several groups in the field of vision
++ = field of vision full of cocci

DISCUSSION

Several investigators have claimed that aureus strains usually disappear within 24 hours in mixed broth cultures with E.coli (94, 114). However, they did not use selective culture mediums to establish the micrococci in their studies, so that a very large surplus of rods may have prevented the verification of the micrococci. However, in the present work the inhibition of the

micrococcal reproduction was only in exceptional cases so severe that the micrococci could have been verified only on selective culture mediums and in every case, even after 5 days incubation, there were living micrococci, which could be shown on selective culture media. Reginer and coll. (197) suggested that the effect of E.coli on aureus is only visible after the logarithmic phase, and that the larger the concentration of the aureus in proportion to that of E.coli the smaller is the inhibitory effect. This finding concurs closely with that obtained in the present investigations, for a comparison of the results obtained with pure cultures shows that the reduction proper in reproduction or the direct destruction of the living cells occurs between the 5-hour and 24-hour growth, i.e. after the logarithmic phase is over, or at the transition. As previously indicated by Fulton (86) pure culture controls are of great importance when studying antagonism in a liquid culture medium, if a truthful picture is to be obtained.

In determining the strength of antagonism, quantitative evaluation is a necessity (118); and it is primarily the number of living bacteria at the various stages that correctly reflects the strength of the antagonism. Hence the cells cannot be directly counted by methods involving staining or other means of computing. When we are investigating a bacterial strain which, like the micrococci, forms clusters difficult to separate, to determine the numbers of living bacteria on the basis of the colonies may give results equalling only one-seventh of the true number (209); the dissimilarity of the results obtained when determinations are repeated may be explicable from these differences alone. On the other hand, it should be borne in mind that, although cultures grown overnight with an identical strain and identical culture medium may be assumed to give an approximately equal amount of living bacteria per volume unit, identical combinations of strains far from always have identical initial conditions in different experiments, especially if the errors due to dilution techniques are taken into account. And as it has been possible to show, when these conditions vary, differences are also found in the strength of the antagonism.

As a suitable method of quantitative determination, either the statistical MPN determination or a colony count by different

methods could have been considered. But, as shown by Worcester (233) — — "if the number is of interest methods more precise than the statistical must be used" — — MPN determinations can hardly be considered suitable. The best results may be assumed to be obtainable by counting the colonies developing on a millipore filter from the desired volume of a suitable dilution of the culture (37, 217). This method in fact proved useful in counting the Escherichia strains but not for counting the micrococci, as even the chromate-containing medium did not prove selective enough when used together with millipore filters: the Escherichia grew on it preventing the identification of the micrococcal colonies. Hence recourse had to be taken to the less accurate system of agar surface count of the micrococci on a selective medium. Especially when a diluent containing potassium chromate was employed for the dilution of mixed cultures, the Escherichiae were incapable of forming macroscopical colonies, although they were nearly a million times more numerous.

A review of the results for individual micrococci and Escherichiae shows that the results very often concur with those obtained previously on solid mediums. However, as shown by Besta and coll. (16), antagonism found on solid mediums and in broth does not always show a parallel course. Thus, in the present investigations with liquid mediums, strains previously identified as weak could prove antagonistic.

Filtrates of E.coli cultures have previously been found to inhibit the growth of aureus strains (18). The present investigations have not corroborated this. The reason may be a dissimilarity of culture medium or, as indicated by Meissner and coll. (170), adsorption of possible inhibitors in the filter.

On the other hand, Barnes (8) reported that filtrates showed no greater inhibition than an equal amount of distilled water. If the inhibitory factors are like colicines, their formation in broth will be in fact almost nil (120), and the best result will be obtained with a thin agar layer. By using the 1.25 per cent agar culture technique (166), filtrates inhibiting the development of micrococci were indeed obtained, although the results could not be reproduced with the same titre values. With sensitive biological methods like the present, however, this cannot be

expected, as even small variations in the conditions may affect the formation of antibiotics.

Gillissen (94) maintained that the antagonism of Escherichia to micrococci was an adaption antagonism. However, he and his co-workers (97) were incapable of showing that mixed culture filtrates had any greater inhibitory effect on the multiplication of micrococci than had the E.coli filtrate alone, as he claims to have found in "coli-typhoid" antagonism. Nor did the present investigations indicate that mixed culture filtrates could have a stronger inhibitory effect than any filtrate whatever. An interesting finding was that Escherichia strain I proved the most sensitive to the inhibitory effect of the filtrates, and that this was primarily auto-inhibition.

In parabiotic determinations, with the strains growing on two sides of a semi-permeable membrane, E.coli has been claimed to inhibit the growth of aureus (228). The result was obtained with a 96-hour E.coli culture. However, the method, claimed to have been discovered by Berdnikov (10) [but the like of which e.g. Frost (85) used at the beginning of the century] failed in the present experiments with this primary incubation to make Escherichia produce enough dialyzable inhibitors inside the bag. By the method described by Heatley and coll. (125), under which the Escherichia strains grow on the exterior surface of a bag hanging in protected air and filled with culture medium, and under which colicines were found already after 24 hours of incubation, a sufficient amount of inhibitors to prevent the growth of all the experimental micrococci employed was found to develop during an incubation period of 72 hours. In keeping with the finding of Wynne and coll. (236), who showed with aerogenes strains that there was a labile antibiotic the effect of which could be inhibited by transferring the bag into a fresh culture medium, the micrococci in the present investigation also grew normally in a batch transferred into an empty bag surrounded by culture medium, although no increase in micrococci was found in the original culture under the influence of Escherichia. That this is not only a question of loss of nutrients is indicated by the fact that the development of the micrococci was comparatively normal also in the batch which was transferred to a bag

surrounded only by saline solution, although the amount of the remaining culture medium was further reduced by diffusion through the membrane. And that the inhibitor involved is extremely labile is shown by the fact that e.g. micrococcal strain 3 developed quite normally on transfer into an empty bag hanging in the air.

III SUMMARY AND CONCLUSIONS

From the investigations described above the following conclusions may be drawn:

(1) The Escherichia strains were antagonistic to the micrococci. However, with the techniques employed, differences in the strength of the antagonism were found between the various strains.

(2) The Micrococcus strains were generally sensitive to the antagonism of Escherichia, but the techniques employed revealed considerable differences in sensitivity among the different strains.

(3) The antagonism of the Escherichia was not correlated with any of their biochemical properties. However, it was found that some of the Escherichia types, when tested by the various techniques, definitely differed from the average antagonistic strength.

(4) The sensitivity of the micrococci was not absolutely correlated with any of their biochemical properties. No correlation was found between the sensitivity of the micrococci to the current antibiotics and to the antagonism of Escherichia. However, certain types clearly differed statistically from the general sensitivity.

(5) Certain strains of Escherichia seemed to possess the ability to produce highly active substances, diffusable in agar, with a specific inhibitory effect on apathogenic micrococci.

(6) Escherichia strains isolated from diarrhoeal stools appeared to have a somewhat stronger antagonistic effect on micrococci than strains isolated from the stools of healthy subjects.

(7) M.pyog.v.aureus types isolated from the same specimens as Escherichia did not seem to possess any greater resistance to the antagonism of Escherichia, than strains isolated separately from Escherichia.

(8) No correlation was observable between the antagonistic

strength of Escherichia and the sensitivity of micrococcus isolated from one and the same specimen.

(9) No correlation was found between the incidence of the micrococci in the stools and their resistance to antagonism.

(10) Micrococci showed an antagonistic effect to Escherichia when challenged by the staled agar technique, and with the same technique an obvious correlation was noted between the antagonistic strength of the micrococci and their resistance to the antagonism of Escherichia.

(11) The inhibitor produced by Escherichia is probably not peroxide.

(12) The resistance of micrococci to the antagonism of Escherichia may result from the enzymes decomposing proteins or other substances produced by them; oxidase formation may also play a part in the resistance.

(13) Primary incubation of Escherichia or the incubation of plates prior to micrococcal inoculation did not seem to have any appreciable effect on the staled agar antagonism. This excludes the effect of nutrient exhaustion as the principal factor under this method.

(14) Antagonism, with staled agar technique, seemed to be primarily bacteriostatic, and only in exceptional cases did extremely sensitive micrococcal strains die within 48 hours on staled agar.

(15) Staled agar plates made of culture medium separated from Escherichia cultures by centrifuging often showed the same inhibitory effect on the growth of micrococci as the plates made of primary cultures. This suggests that inhibitory factors were excreted in the culture medium.

(16) Staled agar plates made of living cells suspended in saline solution seemed as a rule to have a stronger inhibitory effect on the growth of micrococci than plates made of living cells suspended in broth, which indicates that nutrient loss may sometimes have an effect when the staled agar technique is used.

(17) Ethylene oxide appeared to destroy to some extent the inhibitory factors produced by Escherichia.

(18) Escherichia killed by ethylene oxide seemed to provide a good nutrient substrate for micrococci separated from their culture medium.

(19) Inhibitors produced by Escherichia were diffused through cellophane membrane onto solid culture medium.

(20) The agent in Escherichiae inhibiting micrococci is not colicine. In some instances Escherichiae produce some kind of inhibitor acting on micrococci as well as on shigellae.

(21) In liquid culture medium, the numerical ratio of the antagonistic strain and the sensitive strain on inoculation was of importance to the antagonism. This was the stronger the more of the former inoculated compared with the latter.

(22) The antagonistic effect of Escherichia in liquid medium seemed to emerge only towards the end of or after the logarithmic phase. Micrococci did not seem to have any significant effect on the growth of Escherichia in liquid medium.

(23) Filtrates of Escherichia grown in liquid medium showed no definite inhibitory effect on micrococcal cultures, but solutions obtained from agar by deep-freezing, defrosting and filtering seemed to possess antagonistic activity.

(24) Mixed culture filtrates revealed no greater antagonistic effect than filtrates made of pure Escherichia cultures or homologous micrococcal filtrates, irrespective of the technique employed.

(25) When growing on the exterior surface of a semi-permeable membrane bag containing liquid culture medium, Escherichia produced labile inhibitors in the culture medium inside the bag. No inhibitors could be shown when the Escherichia grew in liquid medium outside the dialysis membrane bag.

A study of the antibiosis between the genera Escherichia and Micrococcus reveals that the former seem to have a general antagonistic effect on the latter but that the strength of this effect varies with different strains, and various techniques produce varying results. On the other hand, the micrococci also seem to exert an antagonistic effect on the Escherichia, and this is found especially with strains resistant to the antagonism of Escherichia. The dispersion in both the antagonism and the

resistance of the micrococci is considerably greater than with the Escherichia.

The antagonistic effect of the Escherichia is evidently due mainly to substances produced by them, which can be traced either as diffusing from the colonies into the agar or in centrifuged culture medium. However, they are hardly colicines or any comparable agent. The ability of Escherichia to produce these substances is not decisive, and the manifestation of antagonism also depends on the ability of the micrococci to destroy these substances or inhibit the growth of Escherichia and thus the production of these substances. Nutrient loss may also play a part in the antagonism, especially in minimum conditions, but as long as enough nutrients are available the effect of the loss is obviously negligible. No clear indication of the so-called "direct antagonism" can be shown with certainty.

The verification of antagonism is largely dependent on the technique employed, and different techniques do not yield identical results. Hence a very sensitive biological phenomenon must be involved, significantly affected by even small changes in external conditions.

The behaviour of the Escherichia and micrococci shows a great deal of individualism. Hence no universally applicable rules can be given for the mutual relations of the two genera.

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STUDIES OF THE ACETYLCHOLINE CONTENT AND CHOLINESTERASE ACTIVITY OF THE HUMAN-PATHOGENIC FISH TAPEWORM *(DIPHYLLOBOTHRIUM LATUM)*

BY

O. O. PYLKKÖ

HELSINKI 1956

To the Memory of My Grandmother

PREFACE

I wish to express my sincere thanks to Professor *Armas Vartiainen*, M.D., chief of the Department of Pharmacology of the University of Helsinki. It was upon his suggestion that I undertook the present work, towards which he has shown an unfailing interest, increasing my knowledge in this field by his kindly and constructive advice.

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Helsinki, February 1956

O. O. Pylkkö

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LIST OF ABBREVIATIONS

ACh	= acetylcholine
AChE	= acetylcholinesterase
anti-ChE	= anticholinesterase
ASa	= acetylsalicylic acid
b_{30}	= μl CO_2 evolved in 30 min.
BeCh	= benzoylcholine
BeChE	= benzoylcholinesterase
BuCh	= butyrylcholine
CbCh	= carbamylcholine
ChE	= cholinesterase
DFP	= diisopropyl fluorophosphate
D. latum	= *Diphyllobothrium latum*
EA	= ethylacetate
MBu	= methylbutyrate
MCh	= acetyl-β-methylcholine
Nu-683	= dimethylcarbamate of 2-hydroxy-5-phenyl-benzyl-trimethyl-ammonium bromide
Nu-1250	= N-p-chlorphenyl-N-methylcarbamate of M-oxyphenyl-trimethyl-ammonium bromide
PrCh	= propionylcholine
pS	= —log molar concentration
R_{30}	= Ringer's solution (table 2)
TA	= triacetin
TB	= tributyrin

INTRODUCTION

The broad tapeworm was one of the earliest human parasites to attract scientific attention. This so-called »fish tapeworm», now classified under the order *Pseudophyllideae* of class *Cestoda* was termed in the past century *Bothriocephalus* or *Dibothriocephalus latus*, and early in the present century the name of *Diphyllobothrium latum* gradually gained general acceptance among investigators (Lühe 1910).

Owing to its wide distribution the fish tapeworm occupies the most prominent position among human intestinal parasites in Finland. It became an object of medical investigation in Finland already in 1887, when Runeberg pointed out its possible significance in the etiology of pernicious anaemia. Numerous descriptions and maps have been published concerning its occurrence among the Finnish population (Sievers 1903, 1905, Ehrström 1926, Seppä 1927, Ollilainen 1943, Tötterman 1944, Gylling 1949, Huhtala 1950, Venho and Venho 1953). It was presumed by Ehrström (1926) that about 20 to 25 per cent of the Finnish population were tapeworm carriers. In later materials the frequency has varied with the different investigators, the number of tapeworm carriers being in various series 11.3 per cent (Seppä 1927), 31.1 per cent (Ollilainen 1943), 14.5 per cent (Tötterman 1944) and 18 per cent (Gylling 1949). More recent reports have concluded that the incidence of the fish tapeworm in Finland is probably about 20 per cent of the population (about 20 per cent according to Huhtala 1950 and 21.7 per cent according to Venho and Venho 1953). In practice this signifies that approximately every fifth person in Finland is suffering from the fish tapeworm infection.

Oleoresin of the male fern (filix extract) is the most common anthelmintic used in Finland. It induces the worm to release its

hold on the intestinal wall. However, therapeutic doses (3.0—6.0 g) frequently produce toxic by-effects in the patient. In 1948 Huhtala collected in Finland 82 male fern intoxications, 42 of which had been fatal. At the present time the latter figure is at least 50 (Huhtala 1956). It is impossible, however, to find out the total number of anthelmintic intoxications, since these drugs are very commonly used by the population without the supervision of a physician. Thus the official statistics for 1936—49 contain only 31 fatal cases of anthelmintic intoxication (Huhtala 1953), acute yellow atrophy of the liver being frequently regarded as the cause of death (Huhtala 1956). Electrocardiographic observations have shown, however, that oleoresin of the male fern also produces changes simulating coronary insufficiency (Halonen and Koskimies 1950).

When we take into consideration the high incidence of fish tapeworm infection in Finland and the doubtless toxicity of the vermifugal drugs, the importance of as thorough knowledge as possible of the biological and biochemical properties of this parasite is evident. The discovery of new, less toxic anthelmintic preparations may thus also be facilitated. It is already known that acetylcholine causes relaxation in, at least, the cat-pathogenic tapeworm (Paasonen and Vartiainen 1956). It therefore could be assumed that acetylcholine, in view of this paralysing action, might prove a serious danger to the existence of the human-pathogenic tapeworm. It thus appeared interesting to study, on the one hand, the possible acetylcholine content of the human-pathogenic fish tapeworm, and, on the other hand, the ability of this parasite to hydrolyze acetylcholine and other choline esters.

THE PROBLEMS

It was the object of the present investigation to study:

(1) Whether the fish tapeworm (*Diphyllobothrium latum*) contains acetylcholine, and if so, in what amounts;

(2) Whether the fish tapeworm contains enzymes capable of splitting choline esters, and if so, whether any of these enzymes is specific against acetylcholine;

(3) Whether purification of the crude enzyme preparation obtained from the fish tapeworm facilitates the further characterization of the behavior of the enzyme.

PART I

ACETYLCHOLINE IN THE FISH TAPEWORM

EARLIER INVESTIGATIONS ON THE PHYSIOLOGICAL SIGNIFICANCE OF ACETYLCHOLINE

Acetylcholine (ACh) has been known since 1867 as a synthetic preparation. It is based on choline, which is present in all tissues and which is formed in the intestinal wall by the destruction of lecithine. The pharmacological effects of choline are in principle similar to those of ACh, although much weaker. Already in 1906 it was observed that acetylization markedly increases the intensity of the effect of choline (Hunt and Taveau). Since ACh has a tendency to be readily hydrolyzed to its structural components, choline and acetic acid, the therapeutic significance of this ester is small. ACh is split at a high rate in alkaline solutions at room temperature. The ester disappears from Ringer's solution in high dilutions at cold temperatures also. Dale (1914) suggested that an esterase is responsible for the rapid removal of active ACh from the blood circulation.

The physiological significance of ACh was clarified in connection with the discovery of the chemical transmission of nervous impulses. Loewi (1921) was the first to study the problem of chemical transmission or mediation by demonstrating the presence of a »vagus substance» in the frog heart. He suggested at first (1921, 1922) that the effect of this substance resembles that of potassium or choline. Later investigations led to the conclusion that it is identical with ACh (Loewi and Navratil 1926 a). This was also

confirmed by the fact that eserine and ergotamine were able to sensitize the frog heart toward the vagus substance and ACh in the same manner (Loewi and Navratil 1926 b).

The rôle of ACh as chemical transmitter of nervous impulses also in warmblooded animals was established in experiments with the dog heart, in which ACh was found to be liberated by stimulation of the vagus (Feldberg and Krayer 1933). It was at first believed that ACh as a neurohumoral transmitter is limited to the autonomic nervous system, and mainly to its parasympathetic part, adrenaline being the chemical transmitter in the sympathetic system. It was further observed that electric stimulation of the motor nerves running to striated muscles causes the liberation of ACh in the muscle (Feldberg 1933, Dale, Feldberg and Vogt 1936). When the superior cervical ganglion of the cat is perfused with Locke's solution containing a small proportion of eserine, ACh appears in the venous effluent during stimulation of the cervical sympathetic nerve (Feldberg and Gaddum 1934). This prompted Feldberg and Vartiainen (1934) to study more closely the site of chemical transmission by ACh in the nervous system. It was concluded by them that the transmission occurs at the ganglionic synapses by the liberation of ACh. This concept, which at present is the generally accepted one, obtained support from the observations by v. Muralt (1937, 1943) that ACh is liberated also in the nerve fibre itself — more explicitly in the medullary sheath of the nerve — when the impulse passes through the fibre.

Occurrence of Acetylcholine in Parasites. — It appears to have been proved that ACh is present in certain parasites, especially in those capable of spontaneous movement. Artemow and Lurje (1941) were able to demonstrate ACh or at least a substance producing the essential physiological effects of ACh in the tapeworms *Taenia crassicollis* and *Dipylidium caninum*. It also has been shown by Bülbring, Lourie and Pardoe (1949) that *Trypanosoma rhodesiense* contains ACh and even is capable of synthesizing this substance, whereas ACh was not found in the erythrocytic stages of malaria parasites.

Since the occurrence and amount of ACh in the fish tapeworm, which is a widely distributed human-pathogenic parasite in Finland, have not been demonstrated (von Brand 1952), the determina-

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Since the occurrence and amount of ACh in the fish tapeworm, which is a widely distributed human-pathogenic parasite in Finland, have not been demonstrated (von Brand 1952), the determina-

tion of the ACh content of the fish tapeworm *Diphyllobothrium latum* (*D. latum*) was taken as the subject of investigation in the present work.

PRESENT INVESTIGATION ON ACETYLCHOLINE IN THE FISH TAPEWORM

Material and Methods

Fish tapeworms (*D. latum*) were used immediately after being expelled from patients by means of a vermifuge, since these parasites rarely release spontaneously their hold on the intestinal wall. The worms were transferred as soon as possible to a 10 per cent solution of trichloracetic acid, in which each worm was homogenized and allowed to stand for 1½ hours with occasional stirring. The extracts were then filtered and the precipitates were successively washed 3—4 times, using each time the same volume of 10 per cent trichloracetic acid as for the extraction. The trichloracetic acid was removed with ether until the extracts were only slightly acid (pH 5.5). The residual ether was allowed to evaporate in vacuum.

The ACh content was determined biologically by the method described by Vartiainen (1934), using strips of the dorsal body wall of the cranial parts of the leech. The strip of back muscle was suspended in 5 cc of oxygenated Ringer's solution of the strength used for frog tissue, and left in the solution for 50—60 minutes until fully relaxed. The solution was then replaced with one containing eserine 1: 200,000, in which the leech muscle was left for a further 15 minutes before testing was begun. The extracts to be assayed and the control solutions of ACh with which they were compared were diluted as required with Ringer's solution eserinized as above but without bicarbonate. Each solution tested was always allowed to remain in contact with the leech muscle for 2 or 3 minutes, during which the contraction was recorded on a slowly revolving drum by a frontal lever. The test solution was removed by washing 3—4

times with 5 cc of clean Ringer's solution. Eserinized Ringer's solution was reapplied for 2—3 minutes before the next test was due. An interval of 15—20 minutes between the tests was usually found to be sufficient for relaxation of the leech muscle; in a few cases up to 50 minutes were required for relaxation. The control solution of ACh, the effect of which was compared with the rate of contraction produced by the test extract, was used in a dilution of 10^{-7}. To prevent non-specific contractions a portion of the tapeworm extract was rendered alkaline with N/3 sodium hydroxide and boiled to destroy the ACh, as done by Bülbring *et al.* (1949). After cooling and neutralization with N/3 hydrochloric acid a control was made of the effect of this portion on the leech muscle.

Results and Discussion

The results obtained in these experiments showed that *D. latum* contains small amounts of ACh. As will be seen from *table 1*, the amount ranged from 4.0 to 12.0 μg per gram of fresh worm tissue.

TABLE 1. *Acetylcholine Content of Diphyllobothrium latum*

Number of Worm Specimens	Amount Used for Extraction g	Worm Extract Dilution	ACh Content of Extracts μg/ml	Total Volume of Extracts ml	ACh Content of Fresh Tissue μg/g
1	1.0	1: 10	0.3	4	12.0
2	1.0	1: 10	0.11	4	4.4
3	1.0	1: 10	0.12	4	4.8
4	19.5	1: 10	0.15	60	4.6
5	20.0	1: 20	0.052	77	4.0

The highest yield of ACh in the extracts was approximately as great as the amount of 0.3 μg per ml of trypanosoma extract obtained by Bülbring *et al.* (1949). It is probable that the ACh in the tissue cells of the fish tapeworm is chiefly present in a bound form, since free ACh is rapidly inactivated by enzymic hydrolysis. It is to be noted that ACh is present in the fish tapeworm in about the same amount as in frog heart (Vartiainen 1934), in which, as is well known, it plays a rôle as chemical mediator of transmission of the nerve impulses.

PART II

CHOLINESTERASE IN THE FISH TAPEWORM

EARLIER INVESTIGATIONS ON THE PROPERTIES OF CHOLINESTERASE

The existence of cholinesterase (ChE) was demonstrated in connection with the study of the physiological action of ACh. Dale (1914) attributed the rapid disappearance of active ACh from the blood circulation to some esterase. Later Abderhalden and Paffrath (1926) were able to demonstrate such esterase activity in the small intestine of horse and pig. In their pioneer studies, Loewi and Navratil (1926 a) not only proved that the vagus substance of frog heart and ACh are identical but also that both substances are hydrolyzed by water and glycerine extracts of frog heart. They suggested that the hydrolyzing agent was an esterase. These investigators (Loewi and Navratil 1926 b) also demonstrated that eserine and ergotamine, which caused sensitization to the vagus substance as well as to ACh *in vivo*, were capable of inhibiting the enzymic hydrolysis of these substances *in vitro*. Galehr and Plattner (1928 a, b) in studying the similarity of vagus substance and ACh found that they were destroyed by defibrinated blood. Plattner and Bauer (1928) found that both the vagus substance and the identical ACh were hydrolyzed by mammalian and frog blood. Further evidence on the enzyme character of the hydrolyzing factor was obtained by several investigators (e.g., Engelhart and Loewi 1930, Matthes 1930). Plattner and Hintner (1930) were able

to demonstrate that this enzyme is present in nearly all animal tissues.

In accordance with the observation that the esterase of horse serum hydrolyzed methylbutyrate (MBu) and tributyrin (TB) at a low rate but the esters of choline, such as butyrylcholine (BuCh) and ACh, at a high rate, the greatest activity being exerted against BuCh, Stedman, Stedman and Easson (1932) called the enzyme »choline-esterase». In this manner they pointed out the difference between this enzyme and the enzymes of the liver and pancreas, as manifested by the difference in substrate affinities. In the same report their first method for purification of ChE was described.

Stedman, Stedman and White (1933) continued their investigations in order to find the specific substrate of the enzyme of blood serum, using ACh, BuCh, MBu and TB as substrates. Of the activity values obtained with several species of animals, those against BuCh were most prominent. Tests with the sera of several human subjects also showed that BuCh was split at the highest rate. More precisely stated, the following ratio was obtained for the hydrolysis of the different substrates, i.e., BuCh : ACh : MBu= 6.0 : 3.4 : 0.15. TB was also hydrolyzed but at a low rate. The authors concluded that there probably were two enzymes of different type in the serum but they did not exclude the possibility that ChE might exert its activity also towards simple aliphatic esters. Since no correlation was observed between the rate of hydrolysis of TB and that of choline esters, it was thought that a third enzyme, probably a lipase, also was involved. They also suggested that the specificity of ChE was not strictly absolute. In developing further the purification of ChE, Stedman and Stedman (1935) found that MBu was split at a relatively lower rate by the purified preparation than by the serum alone when compared with the hydrolysis of choline esters. It was therefore thought that the hydrolysis of MBu was not due to ChE alone. Further evidence for the presence of at least two different types of ChE was obtained by other investigators also (e.g., Shaw 1935). Vahlquist (1935), on the other hand, demonstrated that quinine, atoxyl and eserine were capable of inhibiting the hydrolyzing action of human plasma regardless of whether ACh, TB or MBu was used as substrate.

In accordance with this observation he concluded that there was one enzyme in human plasma which was responsible for the hydrolysis of these different substrates. Thus the hydrolysis of ACh was caused by the same enzyme in blood plasma as that of TB. In other words, this would mean that there is no specific acetylcholinesterase (AChE) in plasma. Easson and Stedman (1937) suggested, however, that Valquist had not been able to demonstrate definitely that ChE was responsible for the hydrolysis of MBu. They presented the opinion that the rate of hydrolysis of MBu in human serum was so low that it would fit within the limits of experimental error. In addition, the results obtained by them were able to confirm that the hydrolytic action of serum varies considerably with different animals. For instance, guinea pig serum was shown to contain ChE which split BuCh, and some other esterase which was able to split MBu, while the low level of hydrolysis of TB seemed to be evidence for the probable presence of a third enzyme. The same authors, however, already placed human serum in a special position in the respect that only one enzyme — ChE — was responsible for its hydrolytic action. The action of this enzyme was consistent with the classical theory of Michaelis and Menten (1913). If always two substrates of the three are tested together and if a single enzyme is responsible for the hydrolysis of two different esters, the rate at which a mixture of the two is hydrolyzed will lie between the rates of hydrolysis of each separately.

Gilman, Carlson and Goodman (1939) termed as »specific» ChE the enzyme which was completely inhibited by eserine in minute amounts, and as »non-specific» ChE that which was inactivated by eserine in higher concentrations only.

The conflicting opinions on the ChE activity of the blood were originally due to the fact that the different functional behavior of the enzymes in the red cells and the serum was not taken into consideration. Alles and Hawes (1940) were the first to demonstrate that the ChE activity of the blood cell preparations was due to the red cells, since washed white blood cells were inactive against ACh. They pointed out that red cells were able to hydrolyze ACh at a higher rate than serum already in low concentration of substrate. Acetyl-β-methylcholine (MCh) was also readily hydrolyzed by red cells, whereas serum showed only negligible activity against

this substrate. Richter and Croft (1942) confirmed the non-specific character of the enzyme in human serum. Evidence for the different behavior of the enzymes of serum and red cells was presented also by Zeller and Bisseger (1943) and Augustinsson (1944, 1945).

Mendel and Rudney (1943 a) were able to demonstrate two enzymes in the animal body capable of hydrolyzing ACh: a »true» ChE acting only against the choline esters, and a »pseudo» ChE capable of hydrolyzing not only choline esters but various non-choline esters as well. True, or specific, ChE was found in, e.g., brain and erythrocytes, while pseudo, or non-specific, ChE was present in, i.a., horse serum and dog pancreas. The authors found a decisive difference between true and pseudo ChE in the respect that true ChE displayed its maximum activity at low concentrations of ACh (less than 3 mg per 100 ml) and was inhibited in high concentrations of the substrate, whereas pseudo ChE attained its maximum activity at high concentrations of ACh (above 300 mg per 100 ml). They confirmed the observation of Easson and Stedman (1937) that an eserine concentration sufficient to completely inhibit the hydrolysis of choline esters was, even when 1000-fold, adequate to inhibit only in part the splitting of aliphatic esters.

In the above mentioned publication Mendel and Rudney (1943 a) expressed, like many other workers, the ChE activity as an arithmetical quotient Q, thus:

$$Q = \frac{[\text{volume of } CO_2 \text{ (in } \mu l)]}{[\text{time (in hr.)}] \times [\text{dry wt. of enzyme preparation (in mg)}]}$$

If ACh and TB were allowed to be hydrolyzed by non-specific or pseudo ChE, for example that of horse serum, the following facts were observed when o n e enzyme was responsible for the hydrolysis: (a) the unaltered $Q_{ACh}/Q_{\text{non-choline ester}}$ ratio within the range of 100-fold to 2000-fold purification; (b) the identical inhibitory effect of eserine on choline and non-choline ester hydrolysis when ChE alone is present; (c) the complete lack of addition of activities when a choline and a non-choline ester are hydrolyzed together.

In studying the properties of specific or true ChE, Mendel and Rudney (1943 a) employed red cells of man and different animals

and brain of mouse and dog as sources of enzyme. The crude ChE preparation obtained from the red cells was able to split ACh at a high rate and, in addition, small amounts of TB and MBu. By purification an enzyme preparation was obtained whose specific substrate was ACh; no activity was seen against other substrates. The brain preparations appeared to be capable of hydrolyzing both ACh and TB, but the hydrolysis of ACh was inhibited by eserine while that of TB was not affected by this inhibitor. From this they concluded that the enzyme which was responsible for the hydrolysis of ACh was not involved in the hydrolysis of TB. When ACh and TB were hydrolyzed together, the activity against the mixed substrates was the arithmetical sum of the activities against the two substrates separately. This was considered to be evidence for the existence of two enzymes in the brain preparation, only one of which was specific. The authors presented in the same paper a simple method for the freeing of red cell ChE from other esterases.

After finding that throughout the animal kingdom the true ChE, in contrast to the non-specific enzyme, displayed stronger activity in low than in high concentrations of ACh, Mendel and Rudney (1944) observed that this property was unstable and faded on descending in the evolutionary scale from the higher to the primitive organisms. They assumed, therefore, that the above mentioned typical relationship between the activity and the substrate concentration was merely a secondary feature of true ChE which was dependent on the physical environment, whereas the substrate specificity was an inborn one not dependent on exogenous factors.

When Marnay and Nachmansohn (1938) and Nachmansohn (1939) had observed that ACh liberated in the central nervous system during the nerve stimulation period is destroyed by ChE during the refractory period, it appeared desirable to employ nerve tissue as a source of ChE in characterizing the enzyme. The first preparation of this kind was obtained from the electric organ of *Torpedo vulgaris* (Nachmansohn and Lederer 1939 a). It was found that 1 mg of the enzyme protein of the electric organ was capable of hydrolyzing 100 mg of ACh in one hour. Since 1 mg of enzyme protein of horse serum was able to hydrolyze only 0.5 mg of ACh in one hour (Stedman and Stedman 1935), one of the most striking proofs had been obtained that the enzyme of nervous tissue

is specific AChE. The same conclusion was also drawn by Langemann (1944).

On the basis of a large series of experiments, Nachmansohn and Rothenberg (1944, 1945) classified the ChE:s into specific and non-specific types, which seemed approximately to correspond to the division into true and pseudo ChE types used by Mendel and Rudney (1943 a). It was found to be characteristic of the esterase of nervous tissue, or specific ChE, (obtained from the brain or electric tissues) that no substrate was split at a higher rate than ACh (Nachmansohn and Rothenberg 1945). Propionylcholine (PrCh) was hydrolyzed at nearly the same or a somewhat lower rate, and BuCh at a considerably lower rate than ACh or hardly at all (in some exceptional cases, however, at a rate which was as much as 19—48 per cent of the rate of ACh hydrolysis). Among the pharmacologically active esters, such as carbamylcholine (CbCh) and MCh, the first mentioned was not split, whereas the latter was hydrolyzed but at a significantly lower rate than ACh. The rate of hydrolysis of MCh usually lay between 30 and 60, when that of ACh is designated as 100. The non-choline esters TB and MBu were not split by the extracts of the electric organ, but 1 to 47 per cent was hydrolyzed by brain extracts of different species of animals. Benzoylcholine (BeCh) was not hydrolyzed by nerve tissue at all.

The esterases of the liver, kidney and pancreas, or non-specific esterases (Nachmansohn and Rothenberg 1945) did not hydrolyze ACh at the highest rate. PrCh and BuCh were split considerably more rapidly. The non-specific esterase of the kidney was able to split BuCh at a definitely higher rate than PrCh and at a 300—400 per cent greater rate than ACh. MCh was hydrolyzed at a low rate by the esterase of kidney and pancreas, while the liver esterase split this compound at a rate comparable to that of the specific enzyme.

In addition, Nachmansohn and Rothenberg (1945) confirmed the finding of Stedman, Stedman and Easson (1932) that the ChE of blood serum split BuCh at a more rapid rate than ACh. They also stressed that the physiological substrates of the esterases of different organs were still unknown and were by no means the same in the different organs. In the opinion of Langemann (1944)

the ChE of striated muscle belongs to the so-called specific type. Nachmansohn and Rothenberg (1945) regarded it as possible that the transmission of impulses in the muscle also is a chemical one, similar to that in the nerve fiber. In accordance with the results obtained by them, they suggested that the esterase of striated muscle (cat and rabbit gastrocnemius, ox heart apex) also was specific ChE, since the enzyme hydrolyzed ACh at the highest rate, although it was capable of hydrolyzing BuCh and MBu comparatively more rapidly than the ChE of electric tissue or red cells.

In his studies on liver esterase, which was classed as non-specific by Nachmansohn and Rothenberg (1945), Sawyer (1945) observed that the liver enzyme of certain rodents — guinea pig and rabbit — readily split BeCh but no ACh. He therefore called this enzyme benzoylcholinesterase (BeChE). Mendel and Gunter found that, contrary to the non-specific ChE, BeChE was resistant to eserine in a concentration of 2×10^{-6} M (Hawkins and Gunter 1946).

In studying the typology of ChE:s, Zeller and Bisseger (1943) suggested the term »E type» for the specific ChE of brain and erythrocytes, and »S type» for the non-specific ChE obtained from the blood serum. When it was found by Bovet Nitti (1947) that the enzyme of snake venom was able to hydrolyze ACh and all the non-choline acetate esters investigated by her, Zeller (1948 a, b) regarded that the choline and non-choline ester hydrolysis is due to the same enzyme and proposed that it should be classified as a third (»C») type of ChE.

The properties of ChE have been investigated also with the aid of the step-photometric method. Abdon and Uvnäs (1937) were able to confirm the earlier observation of Vahlquist (1935) that the enzymic hydrolysis of ACh was consistent with the law of simple and unimolecular reactions. In his electrophoretic studies Augustinsson (1944, 1945) found a further decisive difference between the two main types of ChE. Erythrocyte ChE seemed to be more sensitive to changes in pH than serum ChE. Using MCh and BeCh as substrates, he studied further the difference between erythrocyte and serum ChE (Augustinsson 1946). According to his observations, MCh was a more suitable substrate for erythrocyte ChE, since it was much less attacked by the non-specific enzyme than ACh. BeCh, again, was better suited for serum ChE because

it was not hydrolyzed to the same extent by the red cell enzyme as BuCh, in consistence with the earlier observation of Mendel, Mundell and Rudney (1943) and Nachmansohn and Rothenberg (1945). Some years later Augustinsson (1948) published a large comparative study in which the ChE activity of various animal tissues was discussed in detail and a general classification of the enzymes was made. This was made possible by the use of a large variety of substrates and inhibitors. Employing several esters he also described the enzyme activity as a function of substrate concentration. The difference between the results of several earlier investigators, i.a., Mendel and Rudney (1943), and those of Augustinsson (1948) was due to the different experimental conditions in the determination of ChE activity.

Specificity of Cholinesterases

From the publications reviewed above, the opinion was gained that specific ChE is present in the animal kingdom mainly in the red cells, brain and striated muscle of vertebrates and in snake venom. It has also been found in the parotid glands of rabbit and cow (Mendel and Rudney 1943 b). In the nervous system the superior cervical ganglion, however, showed both specific and non-specific activity (Mendel and Rudney 1944). Evidence has also been obtained that the tissues of certain invertebrates contain specific ChE (Prosser 1946), as will be discussed later. Non-specific ChE, again, has been found chiefly in blood serum and in the liver, kidney and pancreas of certain animals. ChE from the cat pancreas, however, showed specific activity (Mendel and Rudney 1943 b). At least the greater part of ChE in aqueous humor was non-specific (Viikari 1955). It seems to the present writer that the concept of specificity of ChE, whether from the point of view of Mendel and Rudney (1943 a) or of Nachmansohn and Rothenberg (1945), should be accepted with some reservation.

In interpreting the ChE of brain and nerve tissue from the point of specificity, it seems that it should exert its strongest action against ACh and not hydrolyze non-choline esters. The values

reported by Nachmansohn and Rothenberg (1945), when $Q_{ACh} = 100$, indicate, however, that for instance in rat brain $Q_{PrCh} = 104—113$, $Q_{TB} = 41—47$, and $Q_{MBu} = 11$. And Bodansky (1946) demonstrated that mouse brain ChE was able to hydrolyze triacetin (TA). Holton (1948) confirmed this observation by Bodansky and reported that TA was also hydrolyzed by the ChE of snake venom, which is regarded as a specific enzyme. Augustinsson (1948) was able to demonstrate that acetylsalicylcholine and TB were hydrolyzed by dog brain ChE equally rapidly as ACh, and BeCh and ethylacetate (EA) to some degree. The rate of hydrolysis of MCh was nearly one-half of that of ACh and TB. In general the ChE activity of the nucleus caudatus was definitely stronger than that of the cortex. The strongest AChE was obtained mainly from the following sources in the animal kingdom: Dart sac of *Helix pomatia*, nucleus caudatus of the bear, brain of *Labrus*, *Paracentrotus* larvae, and brain of the common ray (Augustinsson 1948) and the electric organs of *Torpedo vulgaris* and *Electrophorus electricus* (Nachmansohn and Rothenberg 1945). The data obtained seem to indicate, however, that the specificity of brain and nerve tissue ChE is not definitely absolute. Attempts to purify brain ChE were not satisfactory (Augustinsson 1948). New attempts (Ord and Thompson 1951) have resulted in a purified brain ChE that does not hydrolyze TB.

ChE of striated muscle is considered to be a specific enzyme since it is able to hydrolyze ACh at the highest rate (Langemann 1944, Nachmansohn and Rothenberg 1945). It is nevertheless able to split BuCh and MCh at a rate which is about one-half of that of ACh hydrolysis (Nachmansohn and Rothenberg 1945). However, ChE from striated muscle of the dog is capable of hydrolyzing BeCh at a two-fold and TB at a more than ten-fold rate as compared with the hydrolysis of ACh (Augustinsson 1948).

The erythrocyte ChE has been regarded above as a highly specific enzyme. The data reported by Nachmansohn and Rothenberg (1945) show that ACh was hydrolyzed at the highest rate by erythrocyte ChE and that the hydrolysis of MCh was about one-half of the hydrolysis of ACh. But we can see that the hydrolysis of TB occurred at a rate as high as one-half of the hydrolysis rate of MCh, and that of MBu again was nearly one-half of the hy-

drolysis rate of TB. BeCh, on the other hand, was split at a very low rate. Even when freed from ali-esterases by the method of Mendel and Rudney (1943), the red cell enzyme was able to hydrolyze TA at a relatively rapid rate (Bodansky 1946). Augustinsson (1948) was also able to demonstrate enzyme activity against aliphatic esters in human and horse red cells. Adams (1949) studied in detail the specificity of red cell ChE using MCh and a large variety of non-choline esters as substrates. His results indicated that the acetyl group plays a major rôle in the configuration of rapidly hydrolyzed substrates. That the approach of the alcohol group to the choline structure is also important seemed proved by the very high rate at which the carbon analogue of ACh, 3 : 3-dimethyl butyl acetate, was hydrolyzed. The approximately similar rates at which 2-ethyl butyl acetate and iso-amyl acetate were hydrolyzed appeared to indicate that the branching at the 2 position is only slightly less favorable than branching at the end of the chain. It was pointed out by Adams that the enzyme preparation of human red cells was slightly active towards TB, and summation experiments with EA seemed to indicate that ChE was responsible. Further summation experiments with EA and iso-amyl butyrate also provided evidence for the capacity of the enzyme to hydrolyze at a slow rate butyrates containing a favorable alcohol group.

Adams (1949) concluded that the terms »true» and »specific» as originally defined are no longer tenable. As far as is known at present, ACh at its peak rate is hydrolyzed nearly twice as rapidly as any other ester by the specific enzyme and appears to be the only substrate exhibiting a humped (Haldane) activity-substrate concentration curve, which, since the peak occurs at a relatively low substrate concentration, would particularly adapt this enzyme to the rapid removal of ACh under physiological conditions. The human erythrocyte ChE, regarded as a highly specific enzyme, is capable of catalyzing the hydrolysis of a large number of non-choline esters.

It was demonstrated by Hawkins and Gunter (1946) that the non-specific enzyme was not responsible for the hydrolysis of ACh *in vivo*. After the injection of 2-hydroxŷ-5-phenylbenzyl trimethylammonium bromide (Nu-683, Hoffmann-LaRoche), which is an inhibitor of non-specific ChE *in vivo*, no symptoms were seen

indicative of the accumulation of ACh. As substrate they used BeCh. Recently, however, it has been considered evident that the use of BeCh alone as substrate for the study of non-specific ChE has led to incorrect conclusions (Mendel and Myers 1955). Levine and Suran (1950) reported that they had found an enzyme in pig serum which was relatively inactive to BeCh and MCh but was capable of hydrolyzing ACh very readily. It therefore seems safer not to rely on »specific» substrates for the identification of ChE. MCh, which has been widely used for the identification of specific ChE, has been found to be hydrolyzed by the non-specific ChE of avian species (Earl and Thompson 1952, Austin and Berry 1953, Myers 1953). Both BeCh and BuCh have occasionally been hydrolyzed also by eserine-resistant esterases (Mendel and Rudney 1944, Koelle 1953, Myers 1953).

Cholinesterase Activity of Worms

As was mentioned in Part I, Artemow and Lurje (1941) were able to demonstrate ACh or at least a substance producing the essential physiological effects of ACh in the tapeworms *Taenia crassicollis* and *Dipylidium caninum*. The present writer has found that *Diphyllobothrium latum* also contains ACh (table 1). Bacq and Oury (1937) were the first to demonstrate a strong choline ester-splitting activity in worms such as *Ascaris megalocephala*, *Distomum hepaticum* and *Lumbricus terrestris*. Pennoit-De Cooman (1940) called attention to the presence of several hydrases in *Cysticercus pisiformis*. In an extensive study concerning the enzyme system of endoparasitic animals, Pennoit-De Cooman and van Grembergen (1942) found significant ChE activity in *Planariae*, *Fasciola hepatica*, *Taenia pisiformis* and *Cysticercus pisiformis*. In his review concerning the distribution of ACh and ChE in invertebrates, Bacq (1941) concluded that in general the motor nerves of worms are cholinergic. This would serve to explain the necessity of ChE as inactivator of the ACh liberated during nerve stimulation.

Bullock and Nachmansohn (1942) found that the flatworms *Protyla* (*Dendrocoelum*) and *Planaria* were capable of hydrolyzing ACh significantly even when decapitated. Prosser (1946) suggested that in several invertebrates, e.g. in the annelids, ACh serves as the

neurohumoral transmitter of the nerve impulse into the muscle. In several cases the eserine was found to potentiate the contraction caused by the stimulus. Baldwin and Moyle (1949) were unable to observe any sensitization of the muscle contraction in the *Ascaris lumbricoides* to ACh by eserine treatment and concluded that the ascaris does not contain ChE. This opinion was confirmed later by the experiments of Bueding (1952), who observed that the hydrolysis of ACh by *Ascaris lumbricoides* was negligible and that *Schistosoma mansoni* and *Litomosoides carinii* were capable of hydrolyzing ACh more definitely and BuCh a little. Since ACh was split at a definitely higher rate than BuCh, the author concluded that the schistosoma and litomosoides homogenates contain AChE. In addition, the enzymic hydrolysis of ACh by schistosoma homogenates displayed its optimum already at a relatively low substrate concentration. The slight hydrolysis of ACh by ascaris homogenates may probably be considered to be within the limits of experimental error.

Physico-Chemical Properties of Cholinesterase

ChE is an enzyme which is generally present in living tissues. Like all enzymes, it is a protein (Bodansky 1938). It is large-molecular and does not pass through dialysis membranes. Glick, Glaubach and Moore (1942) suggested that ChE is bound in the α- and β-globulin fractions of proteins, while Mendel and Mundell (1943) observed that the non-specific ChE of dog pancreas was bound in both the albumin and globulin fractions of pancreas proteins. In the opinion of Faber (1943) and Augustinsson (1944), again, ChE is more closely associated with the albumin fraction. In studying the differences between erythrocyte ChE and serum ChE, Augustinsson (1944) determined their isoelectric points (e-ChE 4.65—4.70 and s-ChE 4.36), which indicate a close relationship between ChE and albumins.

ChE is a substance of fairly high stability, especially in glycerin extracts (Glick 1937). Stedman, Stedman and Easson (1932) observed that it retained its activity for several months if the serum was sterile. Vahlquist (1935) found that plasma ChE stored in a stoppered test tube at room temperature remained active for at least one

week. A sample of horse blood was found by Keilin and Wang (1947) to possess 85 per cent of its ChE activity after storage for 42 years. Loewi and Navratil (1926 a), in studying the effect of heat on the esterase activity of frog heart extracts, found that a decrease began at + 56° C. In their opinion no hydrolysis of ACh occurred at 0° C. The freezing-drying process does not decrease the ChE activity itself (Anfinsen, Lowry and Hastings 1942, Augustinsson 1948). On the other hand, the activity is no longer demonstrable at + 70 to + 75° C (Abderhalden and Paffrath 1926, Kahane and Lévy 1936). Active enzyme powders are obtained by cautious evaporation of blood and tissue extracts (Bernheim and Bernheim 1936), but Stedman, Stedman and Easson (1932) already observed that the activity towards ACh is destroyed by drying with acetone and ether. Strelitz (1944) reported that purified ChE preparation of horse serum remains stable for several weeks when stored in the dry state at a low temperature. The purified enzyme is stable in 0.1-n acetic acid and 0.01-n sodium hydroxide, but is destroyed by 0.1-n sulphuric acid and 0.04-n sodium hydroxide.

The optimum temperature for ChE activity is generally considered to be + 37 to + 40° C (e.g., Ammon 1934, Kahane and Lévy 1936, Mendel and Rudney 1943 a, Augustinsson 1948). AChE is considerably more sensitive to changes in pH than the non-specific enzyme (Augustinsson 1944, 1945). The optimum range for AChE activity is reported to be pH 7.4—8.0 (e.g., Alles and Hawes 1940), complete inactivation occurring at pH 4.5 (Augustinsson 1944). On proceeding towards the alkaline side the activity of the specific enzyme declines earlier than that of the non-specific ChE, as is evident from reports published already before the question of specificity was emphasized (e.g., Stedman, Stedman and Easson 1932) as well as from later publications (Augustinsson 1945). Non-specific ChE is inactivated at pH 2 on the acid and at pH 11 on the alkaline side (Werle and Uebelmann 1938, Strelitz 1944, Augustinsson 1944). The optimum pH for it is 8.0—8.5 (Stedman, Stedman and Easson 1932, Bernheim and Bernheim 1936, Glick 1937, Werle and Uebelmann 1938).

DETERMINATION OF CHOLINESTERASE ACTIVITY

The determination of ChE activity is based on the measurement of the hydrolysis of added ACh. Other choline esters as well as non-choline esters may also be used as substrates in the more detailed characterization of the enzyme. Formerly biological methods were chiefly used for the determination of the residual ACh which has not been hydrolyzed. Chemical methods are more in favor today. The advantages of biological methods are their high sensitivity in determining minute amounts of ester. In the chemical methods less time is required for the determination and they are therefore more useful especially in large serial experiments.

Biological Methods. — In these methods the enzyme is allowed to act during a given time on a known amount of ACh, after which the enzymic process is interrupted. The residual amount of ACh is determined by the use of isolated intestine (Abderhalden and Paffrath 1926), frog heart (Loewi and Navratil 1926 a, b, Engelhart and Loewi 1930, Plattner and Hintner 1930), leech muscle (Minz 1932, Feldberg and Rosenfeld 1933, Viikari 1955), frog rectus (Martini and Torda 1937, Viikari 1955), or by measurement of blood pressure (Danielopolu and Popesco 1946).

Chemical Methods. — In estimating ChE chemically the acetic acid liberated by the hydrolysis of ACh was formerly determined very commonly. This may be done by titrating the liberated acid with an alkaline solution of known concentration. Various indicators have been employed, as for instance phenolphthalein, cresol red, bromthymol blue, and others. This method has been used by, e.g., Stedman, Stedman and Easson (1932), White (1933) and Vahlquist (1935). A more convenient method than the use of indicators is electrometric titration (Oka 1954). According to the literature it seems to be advisable to use a quinhydrone electrode (Scoz and

Cattaneo 1937), glass electrode (Glick 1937, Alles and Hawes 1940, Michel 1949, Oka 1954) or antimony electrode (Delaunois and Casier 1946). Today the colorimetric method has also been recommended in order to determine the ChE activity in certain cases (e.g., Gomori 1953). One of the most convenient methods for following the hydrolysis of choline esters, however, is the gasometric one, using Warburg's apparatus. It appears to be very accurate and rapid, and therefore suitable for use in comparative serial experiments. The method is based on the manometric measurement of the volume of CO_2 evolved from a bicarbonate-containing system by the acetic acid formed in the ester hydrolysis. Ammon (1934) was the first to use this method for following the hydrolysis of ACh. The method has then been widely employed and modified by many other investigators (Mendel and Rudney 1943, 1944, Nachmansohn and Rothenberg 1945, Augustinsson 1944, 1948, Holmstedt 1951, etc.)

Units Used in Expressing ChE Activity. — A very useful unit of the ChE activity is Nachmansohn's Q (Nachmansohn and Lederer 1939 b). This is mg ACh hydrolyzed in 60 minutes by 100 mg tissue at + 20° C. After purifying the enzyme of electric tissue, Rothenberg and Nachmansohn (1947) used A as the unit for expressing ChE activity. This is mg ACh hydrolyzed in 60 minutes by 1 mg protein. The unit Cf chosen by Augustinsson (1944) expresses in μmol. the quantity of ACh destroyed in 60 minutes by 1 mg dry substance. Later Augustinsson (1948) symbolized the ChE activity by b_{30}, which represents the amount of CO_2 in μl evolved during 30 minutes. Augustinsson's b_{30} used by the present writer as a symbol for the ChE activity signifies μl CO_2 evolved in 30 minutes by mg or ml of worm homogenate.

PRESENT INVESTIGATION

METHODS

The ChE activity was measured by the WARBURG manometric method modified by Augustinsson (1948), since this method is regarded as best adapted for large comparative serial experiments. The basis of the method is the manometric estimation of the volume of CO_2 evolved from a bicarbonate-containing system by the acid formed in the ester hydrolysis. Conical flasks, each of 15 — 20 ml volume, with one side bulb, were used. The flask constants were determined by the calibration method, using mercury. The manometers were filled with Brodie's solution containing 23 g NaCl and 5 g sodium choleate in 500 ml water. A few drops of an alcoholic solution of thymol were added and the fluid was colored with methyl violet. The density of the solution is 1.034, and 10,000 mm Brodie corresponds approximately to 750 mm Hg.

The volume of the reaction mixture was 2.00 ml in all experiments. Into the main compartment of each flask 1.60 ml of the substrate solution was measured, and into the side bulb 0.40 ml of the enzyme solution, or 0.20 ml of the enzyme solution and 0.20 ml of the inhibitor solution. These solutions were prepared by dissolving the substrate and enzyme preparations in a bicarbonate-RINGER's solution (table 2). The hydrolysis was carried out at 37.5° $\pm$ 0.05° C in a gas mixture of 95 per cent N_2 and 5 per cent CO_2 by volume. The solutions were saturated with the gas mixture during 5 minutes and the flasks were filled after they had been attached to the manometers. Before the enzyme solution was mixed with the contents of the main portion of the flask the temperature equilibrium was attained by shaking in the thermostat for 10 minu-

tes (80—90 oscillations per minute, shaking amplitude about 7 cm). The shaker was stopped and the first manometer was read, lifted from its mount, the contents mixed at zero time, and the manometer placed back on its mount and the shaker started again. At 1 minute intervals the contents of the other flasks were similarly mixed. Usually 8 to 10 manometers were used in each series. Each manometer series was read at 10 minute intervals, with 1 minute between each manometer. Readings were made continuously for 30 minutes. Before each experiment the volume of CO_2 liberated by the tissue homogenate itself (due to possible hydrolysis of endogenous ACh, carboanhydrase activity, changes in pH, etc.) was measured. The latter volume of CO_2 as well as that evolved by the spontaneous hydrolysis of the substrates were taken into consideration in the correction of the values obtained from the readings.

The ChE activity is expressed in µl CO_2 evolved during 30 minutes by mg dry worm homogenate, by ml worm homogenate, or by mg protein nitrogen in the case of purified enzyme preparation. The ChE activity is symbolized according to Augustinsson by b_{30}, which signifies the 30-min. value ($= a_{30}$) minus the amount of CO_2 evolved during the same time period by non-enzymic hydrolysis.

The reliability of the method was determined in preliminary experiments with worm homogenate and enzyme preparation, with each of which 8 determinations were made. The standard deviation obtained with the worm homogenate was 2.72 ml and the standard error of the mean value 0.96. The standard deviation obtained with the enzyme preparation was 2.02 ml and the standard error of the mean value 0.82.[1])

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[1]) The standard deviation S_x of a single observed value x_i is obtained from the following formula:

$$S_x = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}$$

where $\bar{x}$ is the arithmetical mean of the series of observed values and n represents the number of observed values. The standard error of the mean value $S_{\bar{x}}$ is obtained from the formula:

$$S_{\bar{x}} = \frac{S_x}{\sqrt{n}}$$

A glass electrode was used for the pH measurements and indicator papers were also used where great exactness was not necessary. The accuracy of the values obtained with the glass electrode was $\pm$ 0.01 pH unit.

MATERIAL

Ringer's Solution. — All the experiments were carried out in a bicarbonate-Ringer's solution, called R_{30} by Augustinsson (1948). The composition of the solution is given in *table 2*. Fresh

TABLE 2. *Composition of Bicarbonate-Ringer's solution (R_{30})*

Solution	% (w/v)	ml	R_{30} Molarity
NaCl	0.90	100	1.15×10^{-1}
$NaHCO_3$	1.26	30	3.36×10^{-2}
KCl	1.20	2	2.40×10^{-3}
$CaCl_2.6H_2O$	1.76	2	1.20×10^{-3}

R_{30} was prepared daily to prevent deterioration. The pH of R_{30}, after saturation with N_2-CO_2 gas mixture, was 7.40.

Substrates. — The substrates used in this study are listed in table 3, which shows the molecular weights and stabilities of the sub-

TABLE

Substrates	Abbreviation	Formula	M W
Acetylcholine chloride	ACh	$[(CH_3)_3N-CH_2-CH_2-O-COCH_3]$ Cl	18
dl-Acetyl-β-methylcholine chloride (Mecholyl)	MCh	$[(CH_3)_3N-CH_2-CH(CH_3)-O-COCH_3]$ Cl	19
Benzoylcholine chloride	BeCh	$[(CH_3)_3N-CH_2-CH_2-O-COC_6H_5]$ Cl	24
Butyrylcholine chloride	BuCh	$[(CH_3)_3N-CH_2-CH_2-O-COCH_2-CH_2-CH_3]$ C	20
Propionylcholine chloride	PrCh	$[(CH_3)_3N-CH_2-CH_2-O-COCH_2-CH_3]$ Cl	19
Triacetin	TA	$C_3H_5O_3(CO-CH_3)_3$	21
Ethyl acetate	EA	$C_2H_5O-COCH_3$	8
Acetylsalicylic acid	ASa	$HOOC-C_6H_4-O-COCH_3$	18
Tributyrin	TB	$C_3H_5O_3(CO-CH_2-CH_2-CH_3)_3$	30

stances as well as the abbreviations used. Fresh substrate solutions were prepared for each experiment by dissolving the substrate in R_{30} and determining the spontaneous hydrolysis for each separately. A 0.25 per cent solution was employed unless otherwise stated. Experience gained in the course of the investigation sometimes made it advisable to select another substrate concentration. TB emulsions had to be shaken for 1 hour before use.

Preparation of Tissues. — The fish tapeworms needed in the investigation were obtained from the Kivelä Hospital and the Private Clinical Laboratory Minox. The recently expelled worms were rinsed in water and frozen at —20°C in a freezing box. Depending on the experimental conditions, the frozen material was kept for about two weeks, without loss of activity, and then defrozen and minced in R_{30}, employing a test tube homogenizer in most cases. The worms used as material for purification were kept in the refrigerator under toluene for 1 month before preparation (as done by Rothenberg and Nachmansohn 1947). About 1-2 g of worm tissue and 2-6 ml of R_{30} were needed for the preparation of each crude homogenate. If the experiments could not be carried out immediately, the homogenates were stored in the refrigerator for some days without appreciable loss of activity. A total of 1,500 experiments or controls were made with the crude homogenate or the purified enzyme solution. The preparation of the latter is described on page 64.

LE *Substrates.*

	Mol. Wt.	Stability		Manufacturer
		in air	in water	
	181.66	Hygroscopic	Unstable	Hoffmann-La Roche
	195.69	Less hygroscopic	Not very stable	Bios Laboratories, Inc.
	243.73	Non-hygroscopic	Fairly stable	Brit. Drug Houses, Ltd.
C	209.7	Less hygroscopic	Not very stable	Bios Laboratories, Inc.
	195.69	Very hygroscopic	Unstable	»
	218.2		Very stable	Commercial sample
	88.10		Stable	Merck AG.
	180.15	Non-hygroscopic	Fairly stable	Commercial sample
	302.36		Fairly stable	Merck AG.

TABLE 4. *Enzymic Hydrolysis of ACh, MCh, BeCh, and BuCh by Diphyllobothrium*

ENZYMIC HYDROLYSIS OF CERTAIN CHOLINE ESTERS BY *DIPHYLLOBOTHRIUM LATUM* HOMOGENATES

In order to study the ChE activity of *D. latum*, 60 crude homogenates were at first prepared from 15 worm specimens and the hydrolytic power of the homogenates towards ACh, MCh, BeCh and BuCh was tested. Three experiments and one control test were made with each substrate, totaling all together 240 determinations. The values from the 180 ordinary experiments and the mean value for each homogenate are summarized in *table 4*. Non-enzymic hydrolysis has been taken into consideration in the correction of these values. Already before reading of the manometer the amount of CO_2 produced by the crude homogenate itself was eliminated.

It is obvious from the data presented in table 4 that the worm suspensions obtained from *D. latum* show activity towards certain choline esters. The activity was definitely strongest against ACh. MCh was also hydrolyzed, although at a considerably lower rate. BeCh was hydrolyzed by the worm homogenate at least at the same rate as MCh but generally the hydrolysis of BeCh occurred at a somewhat higher rate than that of MCh. BuCh was hydrolyzed at a very low rate or not at all.

In order to compare the hydrolytic action of *D. latum* homogenates against PrCh with that against ACh, 8 suspensions were prepared from 4 specimens. Three experiments and one control test were carried out with each suspension, or a total of 32 determinations. The values obtained from the 24 ordinary experiments and the mean values of each homogenate are shown in *table 5*. From these data it is concluded that ACh was split at approximately twice as high a rate as PrCh.

TABLE 4. *Enzymic Hydrolysis of ACh, MCh, BeCh, and BuCh by Diphyllobothrium latum Extracts.*

μl CO_2 evolved during 30 min. = b_{30}, calculated for 10 mg dry homogenate. Substrate concentration: 0.25%.

No. of Sp.	ACh		MCh		BeCh		BuCh	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	172 157 160	163	32 26 34	31	40 35 23	33	1 5 0.2	2
2	120 112 116	116	20 23 24	22	29 24 17	23	2 1 16	6
3	78 81 83	81	13 12 17	14	25 21 21	22	0 0 0	0
4	70 75 74	73	1 1 3	2	11 16 4	10	0 0 0	0
5	115 110 114	113	16 16 18	17	43 30 32	35	5 5 4	5
6	39 50 41	43	44 4 4	17	13 7 10	10	0 1 0.5	0.5
7	104 102 104	103	10 13 14	12	42 38 35	38	0.7 1.2 2.4	1.4
8	51 41 46	46	5 4 15	8	12 6 9	9	0 11 5	5
9	67 51 —	59	4 — —	4	16 20 —	18	0 0 0	0
10	126 128 122	125	12 16 8	12	34 42 27	34	0 12 12	8
11	118 113 103	111	27 17 17	20	22 21 15	19	0 0 0	0
12	155 149 —	152	27 20 16	21	38 44 39	40	0 0 0	0
13	56 57 64	59	5 6 10	7	24 20 19	21	2 7 0	3
14	70 71 63	68	24 16 7	16	24 33 22	26	5 1 4	3
15	103 100 100	101	14 13 15	14	33 34 —	33	0 0 0	0

TABLE 5. *ChE Activity of Diphyllobothrium latum against ACh and PrCh.* μl CO_2/30 min./10 mg dry homogenate.

No. of Specimens	ACh		PrCh	
	Range	Mean	Range	Mean
1	193 166 195	194	95 106 94	98
2	123 122 122	122	63 70 51	66
3	88 85 88	88	37 40 36	38
4	149 144	147	84 67	75

Marked variations between the individual ChE activity values are apparent from tables 4 and 5. Closer observation of the data indicates, however, that the values obtained for the activity of each worm specimen against the different substrates are proportionate. In other words, when the rate of ACh hydrolysis is a low one, the other choline esters are also split at a relatively low rate. This signifies that the total ChE activity of the homogenate is weak. When, again, ACh is hydrolyzed at a high rate, the hydrolysis of the other choline esters is at a correspondingly high level.

A clearer view of the ChE activity of *D. latum* is obtained from *table 6*. In this table the enzymic hydrolysis of MCh, BeCh, BuCh by suspensions obtained from 19 worm specimens is compared with that of ACh, the latter being expressed as 100.

The values shown in table 6 are evidence in support of the opinion that the ChE of *D. latum* exerts its strongest action against ACh among the substrates employed. PrCh occupies the second position, with a hydrolysis approximately 50 per cent of that of ACh. It may be regarded as probable that the enzyme in the worm homogenate which displays its activity against ACh is identical with that which splits MCh. According to the results obtained in these experiments, the rate of hydrolysis of MCh is about 1 to 28 per cent of the rate of ACh hydrolysis. Experience gained in

TABLE 6. *Enzymic Hydrolysis of ACh, MCh, BeCh, BuCh and PrCh by Diphyllobothrium latum Homogenates.*
Hydrolysis of ACh = 100.

No. of Specimens	ACh	MCh	BeCh	BuCh	PrCh
1	100	20	22	0	
2	100	20	22	2	
3	100	17	26	0	
4	100	1	19	0	
5	100	15	27	4	
6	100	10	28	0	
7	100	12	36	0	
8	100	10	21	10	
9	100	7	30	0	
10	100	11	27	0	
11	100	15	19	0	
12	100	15	25	0	
13	100	11	37	4	
14	100	28	32	4	
15	100	14	34	0	
16	100				51
17	100				54
18	100				43
19	100				51

the course of these experiments seems to indicate that had a young worm or the youngest part of the parasite always been available, the rate of hydrolysis of MCh would have attained, on an average, 20 to 25 per cent of the hydrolysis rate of ACh. Since the rate of MCh hydrolysis is generally known to be considerably lower than that of ACh, a strong ChE activity would be necessary. This is present in the younger tissues of the parasite (table 7). The results obtained supply evidence that the hydrolysis of BeCh takes place at a rate which is 19 to 37 per cent of the hydrolysis of ACh. An evaluation of the BeCh hydrolysis in the light of the ChE activity found in young and older tissues, respectively, indicates that under optimum conditions BeCh would be hydrolyzed at a rate running as high as 30 to 36 per cent of the rate of hydrolysis of ACh. At the same time it may be regarded as probable that the enzyme responsible for BeCh hydrolysis is different from that which splits

ACh and MCh. In practice this would signify that *D. latum* contains at least two different enzymes, i.e., AChE and some pseudo ChE similar to that described by Mendel and Rudney (1943 a) or that which is termed non-specific ChE by Nachmansohn and Rothenberg (1945) and Augustinsson (1944—1948). When we further consider that the hydrolysis of BuCh occurs at a rate which is only 0 to 10 per cent of the hydrolysis rate of ACh (table 6), the presence of a special BeChE in *D. latum* cannot wholly be excluded. Such an enzyme was found by Sawyer (1945) in, for instance, the liver of certain rodents.

TABLE 7. *ChE Activity of Diphyllobothrium latum in Different Parts of the Parasite.* μl CO_2/30 min./10 mg dry homogenate. Substrate: 0.25 per cent solution of ACh.

Part of Parasite	Experiment			Mean
	I	II	III	
Youngest segments	146	147	145	
	144	153	—	147
Younger middle segments	72	77	72	72
	—	—	—	—
Older middle segments	61	64	60	
	68	55	56	59
Oldest segments	50	50	46	50
	—	—	—	—

The ChE activity of different parts of *D. latum* is shown in *table 7*. From 3 to 6 determinations were made from four different parts of the parasite, i.e., youngest segments, younger middle segments, older middle segments, and oldest segments. ACh was employed as substrate. It is seen that a considerably stronger ChE activity is present in the youngest and the younger middle segments of the parasite than in the adult tissues. The results obtained supply evidence that the ACh-splitting power of the youngest tissues of the parasite is more than twice as strong as that of the younger middle segments and nearly three times as strong as that of the oldest tissues of the worm. The differences seen in tables 4, 5 and 6 in the ChE activity of the different worm specimens may therefore

be regarded as due to the part of the parasite which in each case was available for preparation of the suspension. The age of the worm tissues employed apparently had a similar effect on the rates of hydrolysis of MCh, BeCh, BuCh and PrCh.

CHOLINESTERASE ACTIVITY AS FUNCTION OF SUBSTRATE CONCENTRATION

Consistent with the theory of Michaelis and Menten (1913) it is generally accepted that an enzyme will combine with the substrate to form an unstable intermediate enzyme-substrate complex, which then is decomposed into the enzyme and reaction products. This theory has later also been applied to the study of the enzymic hydrolysis of ACh. The influence of the substrate concentration on the reaction velocity has been a subject of investigation. The earlier investigations have generally been carried out with enzyme preparations obtained from blood serum. The dissociation constant of the supposed intermediate compound ACh-ChE was determined for human serum by Glick (1937), for dog serum by Eadie (1942), for human and dog serum by Wright and Sabine (1943), and for dog serum by Goldstein (1944). In all these cases the so-called non-specific enzyme has been studied.

The study of the activity-substrate concentration relationship has since then been regarded as advisable for the characterization of the ACh-splitting enzymes. Alles and Hawes (1940, 1944) were already able to demonstrate that the ChE activity of red cells is depressed by an excess of ACh, which is not the case with the serum ChE. Mendel and Rudney (1943 a) seemed to regard at first the activity-substrate concentration relationship as an argument for the specificity of ChE. After finding that »true» ChE, in contrast to the non-specific enzyme, exerts a stronger activity in low than in high concentrations of ACh, it was suggested by Mendel and Rudney (1944) that this property was unstable and faded on descending in the evolutionary scale from the higher to the primitive organisms. Therefore the typical relationship between activity and substrate concentration would in their opinion

be only a secondary feature of »true» ChE. This, however, does not seem tenable in the case of *D. latum* ChE when ACh or PrCh is used as substrate. In studying the ChE activity of the blood of *Helix pomatia*, Augustinsson (1946) for his part observed that the hydrolysis was depressed by excess of substrate when using ACh but not when MCh was employed. Hawkins and Mendel (1946) represent the opinion that the shape of the activity-substrate concentration curve cannot serve as a basis for distinguishing specific from non-specific ChE.

It is considered by many investigators, however, that the activity-substrate concentration curve has a given shape depending on the nature of the enzyme which in each case is responsible for the hydrolysis of ACh. In his detailed study Augustinsson (1948) described the relationship between substrate concentration and ChE activity by using various choline esters and ChE preparations from different sources. His results seem to provide conclusive evidence that serum ChE, which generally has been regarded as a non-specific enzyme, will give a nearly S-shaped activity-substrate concentration curve. Erythrocyte ChE, on the other hand, which represents the so-called specific type, had a bell-shaped (*Haldane*) activity-substrate concentration curve. The relationship was most evident when ACh was used as substrate. When using MCh a rather bell-shaped curve was obtained with erythrocyte ChE, although it was not as symmetrical as that obtained with ACh. Furthermore, Adams (1949) stated that ACh was hydrolyzed at least twice as rapidly by the specific red cell enzyme as any other ester used by him, and appeared to be the only substrate exhibiting a humped activity-substrate concentration curve, the peak of which occurred already at a relatively low concentration of substrate.

In studying the ChE activity of certain endoparasitic animals, Bueding (1952) found AChE in *Schistosoma mansoni* and *Litomosoides carinii*. Using ACh and TA as substrates he also studied the significance of the substrate concentration on the ChE activity shown by the worm homogenates. The hydrolysis of ACh displayed its maximum already at a relatively low concentration, evidence of which was a humped activity-substrate concentration curve. On the other hand, TA was hydrolyzed by the schistosoma

homogenate at a rate increasing parallel with the increase in substrate concentration. These facts, and the low rate at which BuCh was split, gave him reason to conclude that *Schistosoma mansoni* and *Litomosoides carinii* contain the so-called specific ChE. The optimum activity of this enzyme was found to occur at 0.04 M ACh.

In graphs depicting ChE activity the substrate concentration is usually expressed by the symbol pS (= —log molar concentration). The different pS values obtained may be due to different experimental conditions and methods used. Alles and Hawes (1940, 1944) found the optimum activity of red cell ChE to be displayed at an ACh concentration of pS 4.6. A similar value was obtained by Mendel and Rudney (1943 a, 1945) with brain ChE. According to Zeller and Bisseger (1943) the optimum pS with the erythrocyte ChE was 2.35 and with the brain ChE 2.4. According to Nachmansohn and Rothenberg (1945) these values were 2.25 and 2.0, and to Augustinsson (1946) 2.6 and 2.5, respectively. The presence of potassium chloride seems especially to have an effect on the relationship between ChE activity and substrate concentration, in the manner that KCl reduces the activity at low and increases it at higher concentrations. It was also found that protamine altered the relationship between the specific brain ChE activity and the substrate concentration in the manner that the enzyme displayed its optimum activity at a high substrate concentration (Hawkins and Mendel 1946). This was observed to be tenable also in the case of erythrocyte ChE (Augustinsson 1948).

The activity-pS curves for the enzymic hydrolysis of ACh, PrCh and BeCh by *D. latum* homogenate are shown in *fig. 1*. The b_{30} on the ordinate is plotted against pS on the abscissa, and each point is the mean of two determinations. The ChE of the crude homogenate displayed its optimum activity against ACh at pS 2.0. The second position in the rate of hydrolysis was occupied by PrCh, which at its peak rate was hydrolyzed also at pS 2.0. The activity curve for the hydrolysis of ACh is clearly bell-shaped. Similarly, the activity-pS curve for the hydrolysis of PrCh is almost humped and would seem to indicate that the same enzyme was responsible for the hydrolysis of PrCh, but the curve for BeCh did not attain a definite peak. The optimum pS at which this substrate was hydrolyzed ranged from 2.5 to 3.0, which did not wholly

exclude the possibility that BeCh was hydrolyzed by the same enzyme as ACh and PrCh. The shape of the activity-pS curve for the BeCh hydrolysis did not, however, indicate that only the non-specific ChE would be responsible for its hydrolysis. If it were

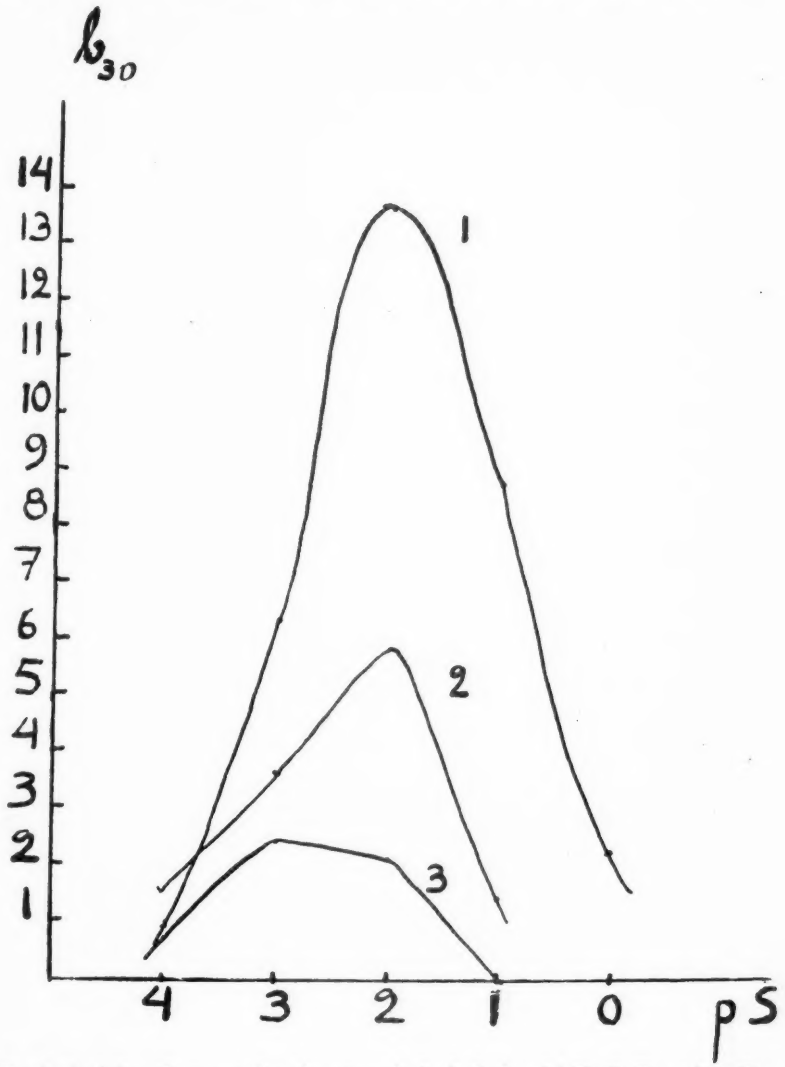

Fig. 1. Activity-pS curves for the enzymic hydrolysis of ACh, PrCh and BeCh by Diphyllobothrium latum homogenate.
1. ACh 2. PrCh 3. BeCh

responsible alone, the activity-pS curve should be S-shaped. The use of eserine as inhibitor, which will be dealt with in a later chapter, will throw further light on this problem. A special BeChE, which was found by Sawyer (1945) in the liver of certain rodents, has proved to be eserine-resistant (Hawkins and Gunter 1946).

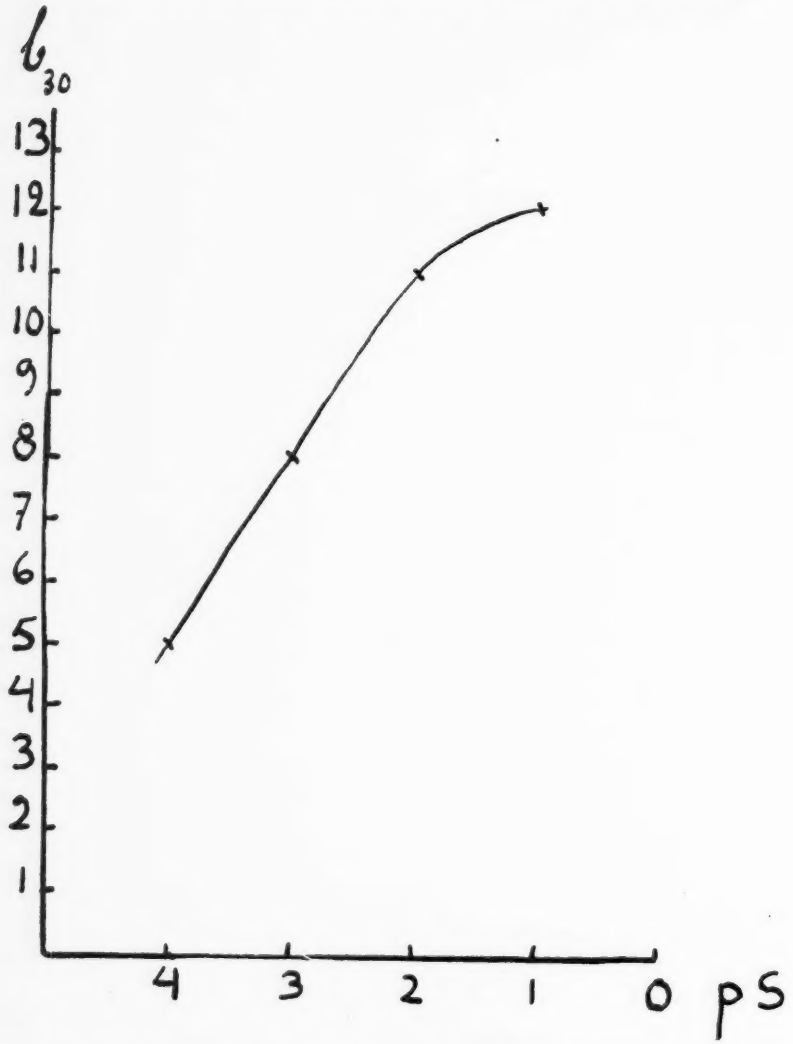

Fig. 2. Activity-pS curve for the enzymic hydrolysis of MCh by Diphyllobothrium latum homogenate.

The activity-substrate concentration curve for the hydrolysis of MCh is shown in *fig. 2*. The b_{30} on the ordinate is plotted against pS on the abscissa. Each point is the mean of two determinations. The ChE of the crude homogenate of *D. latum* displayed its optimum activity towards MCh at pS 1.0. The shape of the curve does not exclude with certainty the possibility that the rate of MCh hydrolysis increases with increasing substrate concentrations. Neither does it show that the same enzyme would not be responsible for the hydrolysis of both MCh and ACh. The optimum pS of MCh is situated more to the right as compared to that of ACh, being 1.0 instead of 2.0. This relationship is similar to that seen by Augustinsson (1948) in respect to erythrocyte ChE, which displayed its optimum activity against MCh at pS 1.5 and against ACh at pS 2.55.

ENZYMIC HYDROLYSIS OF CERTAIN NON-CHOLINE ESTERS BY *DIPHYLLOBOTHRIUM LATUM* HOMOGENATES

The pioneers of ChE investigations, Stedman, Stedman and Easson (1932), already employed in addition to choline esters the non-choline esters MBu and TB in studying the properties of serum ChE. Using the same substrates, Stedman, Stedman and White (1933) pointed out that serum ChE might also attack aliphatic esters. Ten years later Mendel and Rudney (1943 a) provided evidence that the ChE of serum and pancreas is of non-specific nature, since the enzyme was able to hydrolyze ACh as well as TB, and since the hydrolysis of both ACh and TB was inhibited by the same concentration of eserine. They were able further to demonstrate that one and the same enzyme was responsible for the hydrolysis of both substrates, since no addition of activities was observed when the substrates were hydrolyzed together. On the other hand, the authors concluded that brain suspensions contain specific ChE and some other type of enzyme, since the eserine concentration which was capable of inhibiting the hydrolysis of ACh by the brain extracts had no effect on that of TB. And when the brain suspension was allowed to hydrolyze a mixture of the two substrates, an addition of activities was seen. Non-choline esters were also employed by Augustinsson (1948) for characterization of ChE preparations from different sources. They were also used by Adams (1949) in investigating the specificity of red cell ChE.

Bueding (1952) used TA in studying the specificity of ChE of *Schistosoma mansoni*. It was shown that the enzyme of schistosoma homogenate, which was regarded by him as specific, was capable of hydrolyzing TA at an increasing rate with an increasing sub-

strate concentration. Quantitatively the hydrolysis of TA occurred, of course, at a considerably lower rate than that of ACh during the same time period.

The enzymic hydrolysis of TA and EA by *D. latum* homogenate is shown in *tables 8* and *9*. TA was hydrolyzed at a relatively low rate, the hydrolysis being of the same order of magnitude as that of MCh. The results obtained provided evidence that the

TABLE 8. *Enzymic Hydrolysis of ACh and TA by Diphyllobothrium latum Extracts.* μl CO_2 evolved during 30 min. = b_{30}, calculated for 1 mg dry homogenate. Substrate concentration: 0.25%.

No. of Specimens	ACh				TA			
	Experiment			Mean	Experiment			Mean
	I	II	III		I	II	III	
1	2.5	2.4	2.5	2.5	0.8	1.0	1.1	1.0
2	14.4	14.9	—	14.7	0.7	1.2	—	1.0
3	6.6	6.7	—	6.7	0.9	0.8	—	0.9
4	7.3	7.4	—	7.4	1.5	1.6	0.9	1.3

TABLE 9. *Enzymic Hydrolysis of ACh and EA by Diphyllobothrium latum Extracts.* μl CO_2 evolved during 30 min. = b_{30}, calculated for 1 mg dry homogenate. Substrate concentration: 0.25%.

No. of Specimens	ACh				EA			
	Experiment			Mean	Experiment			Mean
	I	II	III		I	II	III	
1	11.4	11.4	11.0	11.3	0.4	0.6	—	0.5
2	6.6	6.7	—	6.7	0	0	0	0
3	7.3	7.4	—	7.4	0.3	0.6	0.3	0.4

hydrolysis of EA occurred at a rate approximately similar to that of BuCh.

The enzymic hydrolysis of acetylsalicylic acid (ASa) by worm homogenate, as compared to that of ACh, is shown in *table 10*. The rate of ASa hydrolysis may be regarded practically as 0.

TABLE 10. *Enzymic Hydrolysis of ACh, and Acetylsalicylic Acid (ASa) by Diphyllobothrium latum Homogenate.*
μl CO_2/30 min. = b_{30}, calculated for 1 mg dry homogenate. Substrate concentration: 0.25%.

No. of Specimens	ACh				ASa			
	Experiment			Mean	Experiment			Mean
	I	II	III		I	II	III	
1	11.4	11.3	11.0	11.2	0	0	0	0
2	5.5	5.7	—	5.6	0	0.1	—	0.08

The results obtained when using TA, EA and ASa seem to provide evidence that *D. latum* homogenates do not contain common esterase, thus confirming the opinion that the *D. latum* ChE has the strongest affinity to ACh. In addition, it is evident that TA and EA are split at a low rate by the same enzyme which hydrolyzes ACh. The relationship between activity and substrate concentration, however, is different from that seen with ACh (*fig. 3*). The ChE activity of *D. latum* was depressed by excess of substrate when ACh was used but not when TA or EA was employed.

The summation experiments with ACh and TB also seemed to be of interest in studying the enzyme activity of *D. latum*. When using the same substrate concentration as Mendel and Rudney (1943 a) it was observed that ACh as well as TB were hydrolyzed by the tapeworm homogenate (*table 11*) and ACh was split at a higher rate than TB. When the worm homogenate was allowed to hydrolyze a mixture of the substrates, an addition of activities was observed: The rate of hydrolysis of the mixture was nearly equivalent to the arithmetical sum of the individual hydrolysis rates of the two substrates. If the same enzyme were responsible for the hydrolysis

TABLE 11. *Enzymic Hydrolysis of Mixed Substrates by Diphyllobothrium latum Homogenate.*

μl CO_2/30 min./0.4 ml crude suspension.

Experiment	0.003 M ACh	0.003 M TB	ACh + TB
1	109	18	132
2	123	22	131
3	119	8	127
Mean	117	16	130

of both substrates, no addition would occur. The results support the assumption that *D. latum* contains, in addition to the ACh- and BeCh-splitting enzyme, also some enzyme of lipase type, possibly tributyrinase.

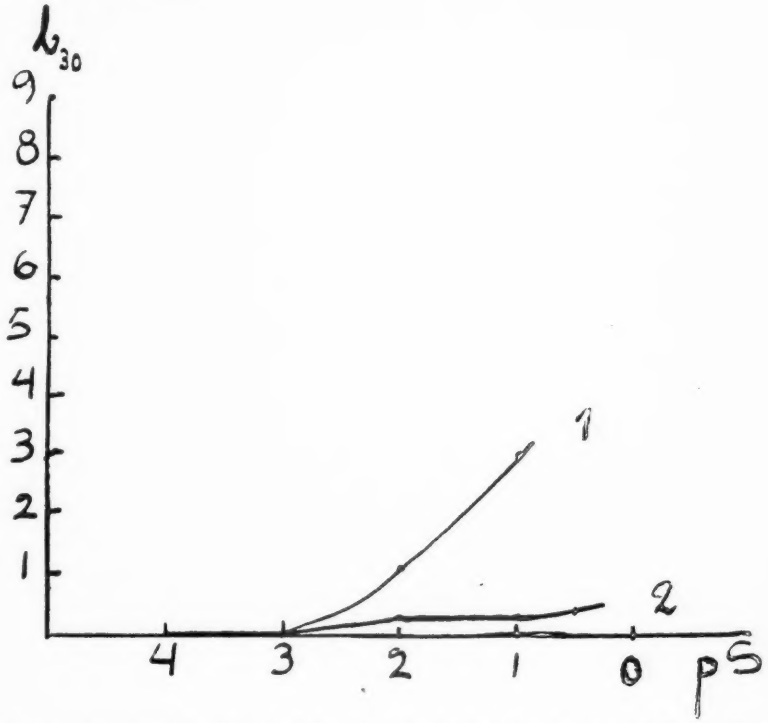

Fig. 3. Activity-pS curves for the enzymic hydrolysis of TA and EA by Diphyllobothrium latum homogenate.

1. TA 2. EA

PART III

EFFECT OF ANTICHOLINESTERASES ON THE CHOLINESTERASE ACTIVITY OF THE FISH TAPEWORM

A. Experiments with Eserine

The term anticholinesterase (anti-ChE) is used to designate drugs whose pharmacological effect is based on the inhibition of ChE activity. The oldest and most potent of these substances is doubtlessly physostigmine, or eserine. Loewi and Navratil (1926 b) found that minute amounts of eserine were already capable of inhibiting the ACh-destroying power of frog heart extract. Evidence of the anti-ChE effect of this alkaloid was later obtained also by Engelhart and Loewi (1930) and Matthes (1930). Stedman and Stedman (1931) presented the opinion that the anti-ChE action of eserine is due to the urethane group. On this basis, synthetic urethanes possessing a similar, although much weaker effect than eserine have been developed. Augustinsson (1948) was able to demonstrate that 10^{-6} M eserine protects ACh from enzymic hydrolysis, and that the action of both the specific and the non-specific ChE is completely inhibited by 3.63×10^{-6}M eserine. The enzyme is not destroyed by eserine but combines reversibly with the drug.

The ChE activity of *D. latum* homogenate was strongly inhibited by 10^{-6} M eserine, as shown in *table 12*, when ACh, MCh, BeCh or PrCh was used as substrate. Suspensions prepared from both the younger segments and the older segments of the para-

TABLE 12. *Influence of 10-6 M Eserine on the Enzymic Hydrolysis of ACh, MCh, BeCh, and PrCh by Diphyllobothrium latum Homogenate.*
b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate	Without Eserine		With 10-6 M Eserine		Inhibition %
	Range	Mean	Range	Mean	
ACh	130 147 134	137	2.3 7.8 4.8	5.0	96.3
	50 52	51	3.5 4.5	4.0	92.2
MCh	4.4 1.5 3.8	3.2	0 0.2 0.9	0.4	87.5
BeCh	19.5 19.3 17.2	18.7	0 2.1 10.4	4.2	77.5
PrCh	29.4 33.1	31.3	7.9 7.3	7.6	75.7

site were allowed to hydrolyze ACh. For the hydrolysis of other choline esters the younger segments of the middle part only were used. The hydrolysis of ACh was practically completely inhibited, i.e., 92.2 to 96.3 per cent. MCh was protected from hydrolysis to a considerable extent, or 87.5 per cent. The inhibition percentages for BeCh and PrCh were 77.5 and 75.7 per cent, respectively. The inhibition percentages in table 12 were obtained from the means of b_{30} values.

B. Experiments with Caffeine

According to Zeller and Bisseger (1943), caffeine inhibits the action of E-type ChE, but that of S-type is not significantly affected. In preliminary experiments it was found that 0.002 M caffeine was not capable of inhibiting the hydrolysis of ACh and BeCh caused by *D. latum* ChE. Therefore 0.02 M caffeine was used as inhibitor. From the data shown in *table 13* it will be seen that the

TABLE 13. *Influence of 0.02 M Caffeine on the Enzymic Hydrolysis of ACh, MCh, BeCh and PrCh by Diphyllobothrium latum Homogenate.*
b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate	Without Caffeine		With 0.02 M Caffeine		Inhibition %
	Range	Mean	Range	Mean	
A C h	104 101	102.5	21.2 31.1	26.2	74.4
M C h	29.6 31.3 29.7	30.2	17.1 22.9 18.2	19.4	35.8
B e C h	66.0 70.7 67.3	68.0	37.7 37.7 50.7	42.0	38.2
P r C h	51.0 42.3	46.7	20.8 27.1	24.0	48.6

hydrolysis of ACh was relatively strongly inhibited by this concentration. The second position in the degree of inhibition was occupied by the PrCh hydrolysis, which probably may be attributed to the same enzyme which is responsible for ACh hydrolysis. The inhibition of MCh hydrolysis by 0.02 M caffeine was about one-half of that of ACh hydrolysis. The inhibition of BeCh hydrolysis, again, was as much as 50 per cent of the inhibition of ACh hydrolysis. The latter finding would be of importance only if BeCh could be regarded as the most useful substrate for the identification of the non-specific ChE. Recently it has been evident, however, that the use of BeCh for this purpose may lead to incorrect conclusions, at least concerning the presence of pseudo ChE in the tissues of ruminants (Mendel and Myers 1955). The inhibition percentages in table 13 were obtained from the means of b_{30} values.

C. Experiments with Dimethylcarbamate of 2-Hydroxy-5-Benzyl-Trimethyl-Ammonium Bromide (Nu-683)

Using BeCh as substrate, Hawkins and Gunter (1946) were able to demonstrate *in vivo* a selective inhibition of pseudo ChE by Nu-683 (Hoffmann-LaRoche). In accordance with this finding

it seemed to be of interest to study if Nu-683 would be able to protect BeCh from the hydrolysis caused by *D. latum* homogenate *in vitro*. In preliminary experiments it was found that the fish tapeworm ChE was not significantly inhibited by 2.5×10^{-8} M Nu-683, and with 10^{-7} M Nu-683 an inhibition of 19.4 per cent only was observed when using ACh and of 16.2 per cent when BeCh was employed. Therefore, an 2.5×10^{-6} M solution of Nu-683 was used in the further experiments. It was found, as shown in *table 14*, that the hydrolysis of BeCh was inhibited most strongly,

TABLE 14. *Influence of 2.5×10^{-6} M Dimethylcarbamate of 2-Hydroxy-5-Phenyl-Benzyl-Trimethyl-Ammonium Bromide (Nu-683, Hoffmann-LaRoche) on the Enzymic Hydrolysis of ACh, MCh, BeCh, and PrCh by D. latum Homogenate.*

b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate	Without Nu-683		With 2.5×10^{-6} M Nu-683		Inhibition %
	Range	Mean	Range	Mean	
A C h	73.7 74.6 67.7	72.0	15.8 18.5 23.0	19.4	73.1
M C h	20.6 18.5 25.8	21.6	13.0 11.5 18.9	14.5	32.9
B e C h	51.4 49.5 55.3	52.1	9.5 4.2 6.4	6.7	87.1
P r C h	51.4 32.1 30.0	31.1	9.5 6.8 5.6	6.2	80.1

or 87.1 per cent. This observation is consistent with those of Hawkins and Gunter and gives support to the assumption that *D. latum* may also contain some pseudo ChE which hydrolyzes BeCh, in so far as the latter may be considered a useful substrate for the indentification of non-specific ChE (Mendel and Myers 1955). The inhibition percentages for ACh hydrolysis (73.1 per cent) and PrCh hydrolysis (80.1 per cent) can only be regarded as evidence that, at least *in vitro*, Nu-683 is not a selective inhibitor for BeCh hydrolysis. A slight inhibition of MCh hydrolysis, on

the other hand, appears to indicate that Nu-683 may inhibit the action of pseudo ChE more strongly than that of true ChE.

D. Experiments with N-p-Chlorphenyl-N-Methylcarbamate of m-Oxyphenyl-Trimethyl-Ammonium Bromide (Nu-1250)

The inhibitory effect of Nu-1250 (Hoffmann-LaRoche) on the ChE activity of the fish tapeworm is shown in *table 15.* It is noted

TABLE 15. *Influence of 10^{-3} M N-p-Chlorphenyl-N-Methylcarbamate of m-Oxyphenyl-Trimethyl-Ammonium Bromide (Nu-1250, Hoffmann-LaRoche) on the Enzymic Hydrolysis of ACh, MCh, BeCh, and PrCh by D. latum Homogenate.*
b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate	Without Nu-1250		With 10^{-3} M Nu-1250		Inhibition %
	Range	Mean	Range	Mean	
ACh	131.4 175.5 180.6	162.5	39.9 37.7 38.8	38.8	76.1
MCh	62.2 68.0 80.4	70.2	10.6 12.2 17.8	13.5	80.8
BeCh	81.2 79.7 79.3	80.1	6.5 16.1 19.3	14.0	82.5
PrCh	29.4 28.8	29.1	15.4 14.5	15.0	48.5

that the *D. latum* specimen used was more capable of hydrolyzing MCh and less capable of hydrolyzing PrCh than usually was the case. This seems to be due to the use of younger tissues of the parasite than in the preceding experiments. In similarity to its above mentioned derivative, Nu-1250 proved to be a drug with the greatest tendency for the inhibition of BeCh hydrolysis (82.5 per cent), at least *in vitro.* The relatively strong inhibition of MCh hydrolysis by Nu-1250 (80.8 per cent), does not exclude a possibility that this substance can be regarded as an inhibitor of true ChE, as described by Hawkins and Mendel (1949).

E. Experiments with Benzoquinonium

According to Goodman and Gilman (1955), benzoquinonium, or mytolone, possesses an anti-ChE effect which is about one-fourth of that of eserine. In inhibiting the hydrolysis of MCh by *D. latum* homogenate to approximately the same extent as that of ACh, as shown in *table 16*, mytolone seems to be rather an inhibitor for

TABLE 16. *Influence of* 2.5×10^{-4} *M Benzoquinonium (Mytolone) on the Enzymic Hydrolysis of ACh, MCh, BeCh, and PrCh by D. latum Homogenate.*

b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate	Without Mytolone		With 2.5×10^{-4} M Mytolone		Inhibition %
	Range	Mean	Range	Mean	
ACh	104.0 99.0 102.0	102.0	28.7 35.3 31.9	32.0	69.0
MCh	12.4 15.3	13.9	3.1 5.2	4.2	69.8
BeCh	37.0 32.7	34.9	28.3 22.8	25.6	26.6
PrCh	51.0 42.3	46.7	46.5 46.4	46.5	0.4

specific ChE. The results obtained with mytolone also confirm the observation that one and the same enzyme is responsible for the hydrolysis of both ACh and MCh in the tapeworm homogenate. The inhibition percentages in tables 14, 15 and 16 were obtained from the means of b_{30} values.

F. Experiments with Diisopropyl Fluorophosphate (DFP)

It was stated by Hawkins and Mendel (1947) that DFP could be used as a selective inhibitor of pseudo ChE but that a suitable substrate was required. BeCh was recommended by them as such a substrate. ACh was not considered suitable, since it is hydrolyzed by both true and pseudo ChE. On the other hand, it was found by Hawkins and Mendel (1947) that also the true ChE is inhibited

by DFP, although a relatively higher concentration of inhibitor is required than in the case of pseudo ChE. From the data in *table 17* it is seen that DFP exerted its strongest inhibitory effect on

TABLE 17. *Influence of 10-6 M DFP on the Enzymic Hydrolysis on ACh, MCh, BeCh, TB, and PrCh by D. latum Homogenate.*

b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

Substrate 0.003 M	Without DFP		With 10-6 M DFP		Inhibition %
	Range	Mean	Range	Mean	
A C h	108.4 104.3	106.4	29.2 30.7	29.9	71.8
M C h	10.3 9.2	9.7	6.0 6.0	6.0	38.2
B e C h	34.8 32.9	33.8	20.9 19.3	20.1	40.5
T B	20.0 21.3	20.6	13.9 15.7	14.8	28.2
P r C h	79.0 78.4	79.1	43.0 42.3	42.7	46.0

ACh hydrolysis — a finding that does not corroborate the conclusion drawn by Hawkins and Mendel. The fact that BeCh was only partly protected from hydrolysis may justify the conclusion that the non-specific ChE is not at least wholly responsible for BeCh hydrolysis in the worm homogenate. The inhibition by DFP of the hydrolysis of ACh and MCh is of the same order of magnitude as that obtained by Hawkins and Mendel. In Part IV a purification method is described by which the worm homogenate ChE was freed from other, disturbing proteins. The ACh and BeCh hydrolysis caused by this partly purified preparation is inhibited by DFP in the manner that BeCh was more strongly protected from hydrolysis than ACh.

G. Effect of Inhibitors on the Hydrolysis of Mixed Substrates

It was observed above (table 11) that when ACh and TB were hydrolyzed together by the worm homogenate, an addition of

activities was found to occur. It therefore seemed to be reasonable to study the effect of eserine on the hydrolysis of a mixture of these two substrates. According to Mendel and Rudney (1943 a), 0.003 M substrate solutions were again used for this purpose. The results obtained are shown in *table 18*. The hydrolysis of ACh

TABLE 18. *Influence of 10-6 M Eserine on the Enzymic Hydrolysis of Mixed Substrates (ACh + TB) by D. latum Homogenate.*

b_{30} = μl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

0.003 M ACh	ACh + Eserine	0.003 M TB	TB + Eserine	ACh + TB	ACh + TB + Eserine
114.4	6.9	18.8	17.4	126.4	16.4
111.1	6.9	19.2	20.0	128.0	20.2
112.7	6.9	18.5	18.7	127.2	18.3

was inhibited by eserine in the usual manner, but eserine had no effect on the TB hydrolysis. In the mixture of ACh and TB, only ACh was protected from hydrolysis, while the hydrolysis of TB was unaffected. The results are consistent with those obtained by Mendel and Rudney with brain suspension, and permit the conclusion that the enzyme in the worm homogenate by which the hydrolysis of ACh was catalyzed was not that which is responsible for the hydrolysis of TB.

Since it was shown that both BeCh and TB were split by *D. latum* homogenate and since it is evident that pseudo ChE is capable of catalyzing the hydrolysis of non-choline esters (Mendel and Rudney 1943 a), it appeared reasonable to study whether BeCh and TB would be hydrolyzed by the same enzyme. This was made possible by the use of eserine and Nu-683 as inhibitors. From the results reported in *table 19* we are able to see that BeCh was protected from hydrolysis by both eserine and Nu-683, whereas the hydrolysis of TB proceeded unchanged. These results provide evidence that TB is hydrolyzed in *D. latum* homogenate by an enzyme which is not responsible for the hydrolysis of ACh or BeCh. This confirms the opinion expressed above that *D. latum* also contains some enzyme of lipase type.

TABLE 19. *Effect of 10^{-6} M Eserine and 10^{-6} M Nu-683 on the Enzymic Hydrolysis of BeCh and TB by D. latum Homogenate.*

b_{30} = µl CO_2 evolved in 30 min. by 0.2 ml crude suspension.

0.003 M	Without Eserine	10^{-6} M Eserine	Inhibition %	Without Nu-683	10^{-6} M Nu-683	Inhibition %
BeCh	50.1	0.4	99.2	55.9	6.7	88.1
TB	12.3	12.0	0	14.4	14.9	0

PART IV

ATTEMPTS FOR PURIFICATION OF CHOLINESTERASE OF THE FISH TAPEWORM

The finding that the ChE of horse serum showed activity against non-choline esters provided an incentive to Stedman, Stedman and Easson (1932) to attempt purification of the enzyme. The purification method used by them was based on ammonium sulphate precipitation. This method was developed further by Stedman and Stedman (1935), who carried out the ammonium sulphate precipitation in several fractions. The purification process yielded an enzyme preparation by which, e.g., MBu was hydrolyzed at a relatively lower rate than by the crude preparation. The ammonium sulphate precipitation was also employed by McMeekin (1939), who obtained from serum a ChE preparation with a 150-fold stronger activity than that of the original serum. Mendel and Mundell (1943) described a method for the purification of pseudo ChE obtained from dog pancreas. In this method the enzyme was first extracted with water, then precipitated by fractioning ammonium sulphate precipitation, followed by adsorption on kieselguhr and elution with ammonium sulphate. The final preparation, 1 mg of which was capable of hydrolyzing 70 mg of ACh during one minute, was found to be about 2,000 times as active as its source per unit of dry weight. It was pointed out by the authors that ChE is associated with both the albumin and the globulin fractions of the pancreas proteins, but since it was found to be very difficult to purify the globulin part, only the albumin fraction, containing about 1/2 of the esterase activity, was used for further purification.

By using ammonium sulphate precipitation, Strelitz (1944) obtained from horse serum a ChE preparation with 5,000-fold activity as compared with its source. The activity of this purified preparation towards TB was maintained constant when compared to that against ACh, independent of the purification level of the enzyme preparation. This was regarded by Strelitz as evidence of the presence of pseudo ChE in the preparation. On the fractioning ammonium sulphate precipitation are also based those methods used for the purification of specific ChE. Such a method was employed also by Rothenberg and Nachmansohn (1947) for the purification of the enzyme obtained from the electric tissue of the electric eel (*Electrophorus electricus*) and for the purification of rat brain ChE by Ord and Thompson (1951). The first step in the purification method presented by Rothenberg and Nachmansohn (1947) was adapted for use in the purification of *D. latum* ChE. Consequently the ChE preparation obtained was not a single, purified enzyme such as meant by Rothenberg and Nachmansohn, but it does not contain other proteins or disturbing cell particles to the same extent as the crude homogenate.

METHODS

The worm tissue was allowed to remain in the refrigerator under toluene for one month. The exudate from the tissue was discarded. The initial weight of the worm tissue at the beginning of the month was 0.5 kilogram. After removal of the exudate the remaining 360 g of tissue were ground in a Waring blendor, and during the grinding 900 ml of 5 per cent ammonium sulphate were added. For the second extraction two-thirds of this grinding was used, consisting of about 1 liter of solution, to which solid ammonium sulphate was added in sufficient quantity to make the concentration of ammonium sulphate 15 per cent. The precipitate formed was centrifuged and discarded, since it was known that only negligible amounts of ChE are precipitated at a concentration of 15 per cent of ammonium sulphate. To the supernatant solution was added solid ammonium sulphate to increase the concentration to 40 per cent. The precipitate obtained by centrifuging at

16,000 r.p.m. was dissolved in R_{30} (the pH of which, saturated with N_2-CO_2 gas mixture, was 7.4), centrifuged and dialyzed against R_{30} for 1 ½ days. The enzyme activity of the preparation obtained was determined by the WARBURG manometric method described above.

HYDROLYSIS OF CERTAIN CHOLINE ESTERS AND NON-CHOLINE ESTERS BY THE ENZYME PREPARATION

The results obtained with the partly purified ChE preparation of *D. latum* are shown in *table 20*. ACh was again hydrolyzed at the highest rate and the values for MCh, BeCh and PrCh hydrolysis were practically unchanged as compared to the values obtained with the crude homogenate (tables 4 and 5). However, when the

TABLE 20. *Enzymic Hydrolysis of ACh, MCh, BeCh, PrCh, TB, and BuCh by Partly Purified D. latum ChE Preparation.*

b_{30} = µl CO_2 evolved by 0.4 ml enzyme suspension. Substrate conc.: 0.003 M.

No. of Enzyme Samples	ACh		MCh		BeCh		PrCh		TB		BuCh	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	111.1 118.8 110.2	113.4	12.2	12.2	48.3 45.1	46.7			0.6 0.3	0.4	2.7 0.8	1.8
2	120.6 127.6	124.1	17.1 19.4	18.3	73.5 76.5	75.0	96.6 97.5	97.0				
3	120.5 116.3	118.4	17.1 16.9	17.0	58.4 59.1	58.8	85.7 88.7	87.2				
4	119.1 115.4	117.3	14.3 12.7	13.5	52.9 53.5	53.2	77.5 78.7	78.1				
5	120.1 119.1	119.6	18.4 19.9	19.1	59.2 59.8	59.5	81.6 83.0	82.3				
6	132.4	132.4	10.2 15.9	13.0	52.1 60.0	56.1						
7	125.3 125.1	125.2					83.1 93.4	88.3	0 0	0		

activity of the crude tapeworm homogenate was compared with that of the purified preparation obtained from the same worm specimen, the purified preparation displayed in fact a somewhat

stronger ChE activity than the crude homogenate. When using, for instance, ACh as substrate the b_{30} values for the crude homogenate and the purified preparation obtained from the same individual were, respectively, 84.4 and 108.0 µl CO_2 in 30 minutes per 1 mg protein nitrogen. On the other hand, the enzyme preparation showed no activity towards TB, which seems to indicate that the ACh-splitting enzyme is not responsible for the hydrolysis of TB by the crude homogenate. The occurrence of TB hydrolysis in the purified preparation would have been evidence for the presence of some non-specific ChE. The rate of BuCh hydrolysis also showed no increase over the values obtained with the crude homogenate. Consistent with the optimum pS found in the experiments with the crude homogenate, the hydrolysis of ACh by the enzyme preparation attained its optimum at pS 2.0 and was depressed with increasing substrate concentrations *(fig. 4)*. The hydrolysis of BeCh has its optimum at pS 2.5, which does not exclude the possibility that the ACh-hydrolyzing enzyme may also attack BeCh.

It seemed desirable to study also the effect of several inhibitors on the ChE activity of the purified preapration. The results obtained in the inhibition experiments are listed in *table 21*. The hydrolysis of ACh was inhibited by eserine to the same degree as above. The inhibition percentage obtained in using DFP was significantly lower than above, being 54.9 per cent, which seems to be an indication of the specific nature of the ACh-splitting enzyme (Hawkins and Mendel 1947). The inhibition of ACh hydrolysis by Nu-683 was also lower than above, being 61.0 per cent. Mytolone likewise showed a weaker inhibitory effect against ACh hydrolysis than above, the percentage being 37.3 per cent. On the other hand, 5-hydroxytryptamine, which was found by Langemann (1954) to possess anti-ChE activity, did not appear to be capable of inhibiting the hydrolysis of ACh more than 8.5 per cent. However, according to Langemann, 5-hydroxytryptamine exerts its anti-ChE effect in very much higher concentrations than eserine.

The hydrolysis of MCh was inhibited approximately equally well by eserine (94.7 per cent) as by DFP (96.3 per cent), which is based on the fact that DFP also has an inhibitory effect on the

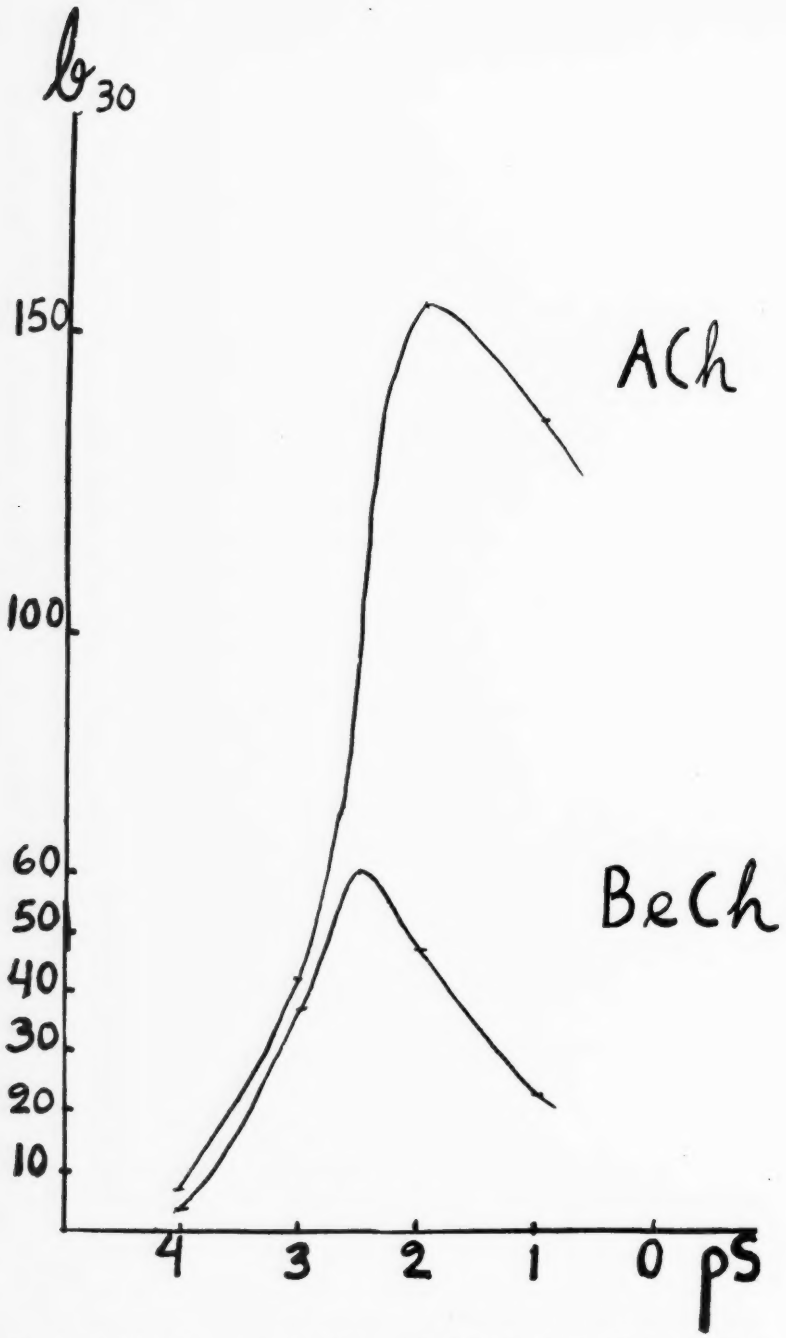

Fig. 4. Activity-pS curves for the enzymic hydrolysis of ACh and BeCh by the enzyme solution.

TABLE 21. *Effect of Several Inhibitors on the Enzymic Hydrolysis of ACh, MCh, BeCh, and PrCh by Partly Purified D. latum ChE Preparation.*
b_{30} = µl CO_2 evolved by 0.4 ml enzyme suspension or 1 mg protein nitrogen. Substrate concentration: 0.003 M.

Inhibitor Concentration	ACh						MCh						BeCh						PrCh					
	No Inhibitor			Inhibitor			No Inhibitor			Inhibitor			No Inhibitor			Inhibitor			No Inhibitor			Inhibitor		
	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N.	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N	Range	Mean	µl/mg prot.N
10^{-6} M	120.6	124.1	112.8	7.8	7.3	6.6	17.1	18.3	16.6	0.9	0.9	0.8	73.5	75.0	68.1	0	0		96.5	97.0	88.2	0	0	
Eserine	127.6			6.8		inh. % 94.1	19.4			1.0		inh. % 94.7	76.5			0		inh. % 100	97.5			0		inh. % 100
10^{-6} M	120.5	118.4	107.6	51.2	53.4	48.6	17.1	17.0	15.5	0	0.6	0.5	58.4	58.8	53.4	3.0	3.9	3.5	85.7	87.2	79.3	20.0	16.3	14.9
D F P	116.3			55.6		inh. % 54.9	16.9			1.2		inh. % 96.3	59.1			4.8		inh. % 93.4	88.7			12.8		inh. % 81.3
10^{-6} M	119.1	117.3	106.6	47.1	45.7	41.5	14.3	13.5	12.3	0	0		52.9	53.2	48.4	5.8	6.0	5.4	77.5	78.1	71.0	29.1	27.4	24.9
Nu-683	115.4			44.4		inh. % 61.0	12.7			0		inh. % 100	53.5			6.1		inh. % 88.8	78.7			25.7		inh. % 64.9
2.5×10^{-4}M	119.1	117.7	107.0	76.2	73.8	67.1	18.4	19.1	17.4	1.0	2.5	2.3	59.8	59.5	54.1	46.0	44.4	40.3	81.6	82.3	74.8	67.3	67.4	61.3
Mytolone	116.3			71.5		inh. % 37.3	19.9			4.0		inh. % 87.0	59.2			42.8		inh. % 25.4	83.0			67.4		inh. % 18.1
5×10^{-3}M	132.4	132.4	120.4	121.1	121.1	110.1	10.2	13.0	11.8	5.5	8.0	7.2	52.1	56.1	51.0	42.0	42.0	38.1						
Oxytryptamine						inh. % 8.5	16.0			10.4		inh. % 39.1	60.0					inh. % 25.2						

action of specific ChE in the concentration used. Non-specific ChE can hardly be responsible for the hydrolysis of MCh in the enzyme suspension. Nu-683 was capable of inhibiting completely (100 per cent) the hydrolysis of MCh by the enzyme suspension, which is not compatible with the opinion expressed by Hawkins and Gunter (1946). The inhibitory effect of mytolone on the hydrolysis of MCh by the enzyme preparation was stronger (87.1 per cent) than that observed with the crude homogenate. Hydroxytryptamine seemed to inhibit the hydrolysis of MCh definitely more strongly (39.1 per cent) than that of ACh hydrolysis.

The hydrolysis of BeCh by the enzyme preparation was completely inhibited (100 per cent) by eserine, which only serves as evidence that ChE is responsible for the hydrolysis of this substrate. DFP also was capable of inhibiting the BeCh hydrolysis rather completely, 93.4 per cent, which was nearly 40 per cent greater than the inhibition percentage obtained for the ACh hydrolysis.

Eserine protected completely (100 per cent) PrCh from hydrolysis caused by the enzyme preparation. The inhibitory effect of DFP was somewhat lower (81.3 per cent). The hydrolysis of PrCh was inhibited by Nu-683 to approximately the same degree (64.9 per cent) as that of ACh. The anti-ChE effect of mytolone on the hydrolysis of PrCh by the purified enzyme preparation was not strong but nevertheless was more definite (18.1 per cent) than on that by the crude homogenate.

The effect of inhibitory substances on the hydrolysis of BuCh was not tested, since it was only negligibly hydrolyzed by both the crude homogenate and the purified preparation.

Summation Experiments with the Partly Purified Preparation. — The ChE activity of the enzyme preparation was studied using mixed substrates (ACh + TB) in concentration 0.003 M. The results shown in *table 22* clearly indicate that no addition of activities occurred, as had been the case with the crude homogenate. This finding confirms the observation reported above (table 20) that the partly purified *D. latum* ChE preparation exerts no activity towards TB.

TABLE 22. *Enzymic Hydrolysis of Mixed Substrates (ACh + TB) by the Purified D. latum ChE Preparation.*

μl CO_2 evolved during 30 min. = b_{30}, calculated for 0.4 ml enzyme suspension and 1 mg protein nitrogen. Substrate concentration: 0.003 M.

Experiment	0.003 M ACh		0.003 M TB	ACh + TB	
	μl/0.4 ml Suspension	μl/mg Prot. N	μl/0.4 ml Suspension	μl/0.4 ml Suspension	μl/mg Prot. N
1	125.3	114.0	0	124.4	113.1
2	125.1	113.7	0	118.8	108.0
Mean	125.2	113.9	0	121.6	110.6

NON-ENZYMIC HYDROLYSIS

In determining ChE activity by biological methods (page 32) strong concentrations of enzyme and relatively low concentrations of substrate are generally used. It therefore has not always been considered necessary to determine the non-enzymic hydrolysis. In estimating ChE activity by chemical methods the enzyme concentration is much lower. Since the values obtained from enzymic hydrolysis may thus be low and in certain cases the non-enzymic hydrolysis forms a considerable part of these values, it has been advisable to determine the latter also when the manometric method is employed, as in the present investigation. The stability of the substrates in air and in aqueous solutions was already given in table 3.

The values for non-enzymic hydrolysis of the several substrates used are shown in *table 23*. In order to estimate the non-enzymic hydrolysis the substrate solution in the main compartment of the flask was mixed with the R_{30} of the side bulb. The non-enzymic hydrolysis for each substrate was determined at + 37.5° C in the two concentrations used during the work (0.25 per cent and 0.003 M), and expressed in µl CO_2 evolved during 30 minutes. Each value

TABLE 23. *Non-Enzymic Hydrolysis of Substrates.*
µl CO_2 evolved during 30 min.

Concentration	ACh	MCh	BeCh	BuCh	PrCh	TB
0.25 %	3.214	0.566	0.400	0	2.0	—
0.003 M	1.0	0.700	0.400	—	0.700	0.400

in the table is the mean of three determinations. It will be seen that the spontaneous hydrolysis of ACh occurred at the highest rate. When a 0.003 M solution was used it was definitely lower than in a concentration of 0.25 per cent. The 0.25 per cent solution of PrCh showed the next highest spontaneous hydrolysis, which again was definitely lower in the 0.003 M solution. The non-enzymic hydrolysis of MCh, on the other hand, was higher in the 0.003 M solution than in the 0.25 per cent concentration. In both concentrations the non-enzymic hydrolysis of MCh was slightly higher than that of BeCh. BuCh was very stable as a solution. The non-enzymic hydrolysis of TB showed the same order of magnitude as that of BeCh.

DISCUSSION

In accordance with the finding that ACh causes relaxation in the cat-pathogenic tapeworm (Paasonen and Vartiainen 1956) it was regarded as possible by the present author that the specific, ACh-decomposing enzyme plays a certain rôle in the kinetics of this parasite. It was not only demonstrated during the present investigation that the human-pathogenic fish tapeworm (*D. latum*) contains ACh in its intact tissues, but also that the tissue homogenates prepared from the parasite are capable of hydrolyzing ACh at the highest rate of all the choline esters used. This ACh-hydrolyzing power was found to be somewhat stronger in the partly purified enzyme preparation obtained from the parasite than in the crude homogenate. Furthermore, the ACh-splitting enzyme of *D. latum* was found to possess also other typical properties usually attributed to the so-called specific ChE (Nachmansohn and Rothenberg 1945, Augustinsson and Nachmansohn 1949): PrCh was hydrolyzed at nearly the same or a somewhat lower rate than ACh. MCh was also split, but at a considerably lower rate, and BuCh was split at a very low rate or not at all. The enzyme showed some or no affinity to non-choline esters. When the enzyme activity was plotted against log M ACh or PrCh concentration a bell-shaped curve was obtained, consistent with the theory of Haldane about inhibition of enzyme by excess of substrate. And the optimum pS for ACh hydrolysis was found to be pS 2.0, which corresponds to the pS value for the specific brain ChE obtained by Nachmansohn and Rothenberg (1945).

It has recently been reported by the present writer (1956) that *D. latum* and *Taenia saginata* contain, in addition to a specific ACh-hydrolyzing enzyme, a second type of esterase with ability

to split BeCh. The author was unable, however, to demonstrate definitely that a non-specific ChE would have been responsible for the hydrolysis of BeCh since the worm homogenates, on the other hand, showed no significant activity towards BuCh. The latter observation has been confirmed in the case of *D. latum* during the present investigation. As, moreover, Cohen, Kalsbeek and Warringa (1949) were able to conclude on the basis of their experiments that the demonstration of the presence of a non-specific ChE should be based on the rate of BuCh hydrolysis, we are not justified in suggesting that a non-specific ChE would wholly be responsible for the BeCh hydrolysis in the *D. latum* homogenate. And, on the other hand, it has recently been stated as evident that the use of BeCh for the identification of non-specific ChE has led to incorrect conclusions (Mendel and Myers 1955). It was therefore assumed that *D. latum* may contain a special BeCh-hydrolyzing enzyme, sensitive to inhibition by eserine, Nu-683, Nu-1250, or DFP. Or it may also be suggested that the BeCh-hydrolyzing power is a special feature of the same enzyme which hydrolyzes ACh, MCh and PrCh and which in view of its other properties has been regarded as specific ChE. The latter opinion was further supported by the observation that the activity-pS curve for BeCh hydrolysis both by the crude homogenate and the purified preparation exhibits also a humped shape, i.e., the enzymic action is inhibited by excess of substrate.

The present writer has demonstrated the occurrence of an eserine-resistant enzyme in the crude homogenate of *D. latum* with ability to split TB. This was revealed in the summation experiments with ACh and TB. In the presence of eserine no addition was observed, since only the TB hydrolysis proceeded unchanged. Neither was TB protected from hydrolysis by Nu-683. It was therefore concluded that TB was not hydrolyzed by the same enzyme(s) responsible for the hydrolysis of ACh or BeCh. It has been stated as evident that polyalkylphosphates are useful tools in distinguishing esterases from lipases, since the latter are relatively resistant to these inhibitors (Webb 1948, Mounter and Whittaker 1953, Alridge 1954, Myers, Schotte and Mendel 1955). As the hydrolysis of TB by crude homogenate of *D. latum* was comparatively resistant also to DFP, it is probably

true that TB was hydrolyzed by some enzyme of the lipase type, possibly tributyrinase. The purified enzyme preparation of *D. latum* showed no activity against TB, which for its part is further evidence that *D. latum* does not contain ChE of the classical non-specific type.

The experiments with enzyme inhibitors showed in addition that the hydrolysis of ACh and MCh by the crude homogenate ChE was inhibited most strongly by eserine. Caffeine, which has been regarded as an inhibitor of »E» type ChE by Zeller and Bisseger (1943), was capable of inhibiting most strongly the ACh hydrolysis by the crude homogenate. And Nu-1250, again, which has been considered by Hawkins and Mendel (1949) to be an inhibitor of true ChE, inhibited relatively strongly the hydrolysis of MCh by *D. latum* homogenate. The hydrolysis of ACh by the partly purified *D. latum* ChE was definitely less inhibited by DFP and Nu-683 than that of BeCh. The latter inhibitors have been regarded as selective in the identification of the non-specific ChE (Hawkins and Gunter 1946, Hawkins and Mendel 1947).

SUMMARY

In Part I of the present investigation the acetylcholine content of the fish tapeworm (*Diphyllobothrium latum*) was assayed biologically, using leech muscle. It can be regarded as evident that the parasite contains small amounts of acetylcholine ranging from 4 to 12 μg per gram fresh tissue.

In Part II the cholinesterase activity of the fish tapeworm was the object of investigation. Using Warburg's gasometric method, the author was able to demonstrate that the parasite contains an enzyme capable of hydrolyzing acetylcholine at the highest rate of all the substrates used. This ACh-splitting enzyme was also found to possess other properties usually regarded as typical for the so-called specific cholinesterase. Propionylcholine was split by it at a rate which was approximately 50 per cent of the rate of acetylcholine hydrolysis, and acetyl-β-methylcholine was hydrolyzed by the enzyme at a rate ranging from 1 to 28 per cent of the rate of acetylcholine hydrolysis. Butyrylcholine was split at a rate ranging from 0 to 10 per cent of the rate of acetylcholine hydrolysis. Non-choline esters were hydrolyzed also at a low rate, or in certain cases not at all. When the enzyme activity was described as function of the substrate concentration, a bell-shaped curve was obtained for the rate of hydrolysis of acetylcholine and propionylcholine, which indicates that the enzyme was inhibited by excess of substrate.

The author was further able to demonstrate that benzoylcholine was hydrolyzed by the *D. latum* cholinesterase at a rate ranging from 19 to 37 per cent of the rate of acetylcholine hydrolysis. It was assumed by the author that the hydrolysis of benzoylcholine is probably due to a special benzoylcholine-splitting enzyme which was shown to be sensitive to eserine, Nu-683,

Nu-1250, or DFP. Or the benzoylcholine-splitting power in *D. latum* may be regarded as a special feature of the enzyme hydrolyzing acetylcholine, acetyl-β-methylcholine and propionylcholine since the benzoylcholine hydrolysis was also inhibited by excess of the substrate. Further the author has found that the crude homogenate of *D. latum* contains some lipase-resembling enzyme with ability to hydrolyze tributyrin.

In Part III the effect of inhibitors on the cholinesterase activity of the fish tapeworm is reported. Eserine, caffeine, Nu-683, Nu-1250, mytolone, hydroxytryptamine and DFP were employed as anticholinesterases. Hydroxytryptamine exhibited a weak, mytolone a relatively strong and all the other inhibitors a strong anticholinesterase activity. Nu-683 was not found to be a highly selective inhibitor of the hydrolysis of benzoylcholine, at least *in vitro*. Nu-1250 could be regarded as a useful inhibitor for identification of the specific cholinesterase in certain cases, also *in vitro*. The hydrolysis of tributyrin was found to be resistant to eserine, and relatively insensitive to DFP.

In Part IV an attempt for the purification of the cholinesterase of *D. latum* homogenate is described. The cholinesterase retained its activity against the cholinesters, and the activity was slightly increased during the purification process. The partly purified enzyme preparation obtained did not contain other proteins or disturbing cell particles to the same degree as the crude homogenate. The acetylcholine hydrolysis by the purified preparation was found to be less sensitive to DFP and Nu-683 than that caused by the crude homogenate. In addition, the author was able to demonstrate that the purified preparation had no affinity to tributyrin.

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